STUDIES ON BETALAIN PHYTOCHEMISTRY BY MEANS OF ION-PAIR COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

Betalains are N-heterocyclic plant pigments and display color shade from yellow-orange to red-purple that replace anthocyanin in certain species of the Chenopodiaceae. Only several betalains are completely understood and available as analytical references due to their high instabilities and rare appearances in a few edible crops. That requires robust techniques applicable for large-scale production and investigation of new betalain resources. Countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC) are preparative purification techniques based on continuous liquid-liquid distribution, which have been intensively applied on natural pigments, such as anthocyanins, carotenoids, chlorophylls and betalains.

This work contributed to the existing phytochemical studies on betalains from several highly pigmented cultivars namely *Opuntia stricta var dillenii*, *Atriplex hortensis var rubra* and *Hylocereus polyrhizus*; the results confirmed the feasibility of lab-scale preparative isolation of pigments using the all-liquid CCC techniques. The application of ion-pair HPCCC on *Opuntia spp.* pigments showed comparable distribution values between analytical and preparative runs. Similar results were seen in the comparison between CCC and CPC experiments, indicating the possibility for scale-up and the re-producibility of these methods. The metabolites of this fruit were investigated next to its pigments, from which betalains and flavonoid glycosides were isolated from unusual cyclic peptides. This work also examined the heat stability of *Opuntia stricta var dillenii* pigments under processing conditions; in which the intact pigments were not well separated from their thermal artifacts.

This study developed a large lab scale pre-purification process applied for complex extracts of betalain plant sources, using the renewable resource of bio-degrade chitin. This approach included a simple adsorption and desorption step based on strong and specific pigment binding capacity to the chitin material. Analytical comparison by LC-ESI-MS analysis was done to optimize the procedure and to prove its capacity to concentrate the minor pigments from heterogeneous betalain crude extracts of *Beta vulgaris*, *Bougainvillea* and *Opuntia spp.* This enrichment approach was then applied to the concentrates of *Atriplex spp*. and *Hylocereus spp.* pigments on larger scale prior to CCC separation. The combination of this chitin process with the CCC separation using ion-pair solvent system TBMe/n-BuOH/ACN/water-TFA monitored by ESI-MS/MS injection profiling successfully fractionated the principle pigments of *Atriplex hortensis* from their flavonoids, and isolated highly pure celosianin II for structural elucidation by 1D/2D-NMR spectroscopic analysis. The ineffective separation of *Hylocereus* pigments indicated high polarities of these betalains structures, which require stronger ion-pair reagents for sufficient fractionation. These results confirmed the indispensable need of ion-pair reagents within CCC separations of such highly hydrophilic betalains structures.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

ACN	Acetonitrile
AcOH	Acetic acid
AmAc	Ammonium acetate
Anal	Analytical
ATPS	Aqueous Two Phase System
Atriplex	*Atriplex hortensis var rubra*
BPC	Base peak chromatogram
CCC	Countercurrent Chromatography
CD_3OD	Deuterated methanol
CPC	Centrifugal Partition Chromatography
C_U	concentration of compound in upper phase
C_L	concentration of compound in lower phase
^oC	Degree Celsius
DA	Degree of acetylation
DAD	Diode Array Detector
DES	Deep Eutectic Solvent
ESI-MS	Electrospray ionization-Mass spectrometry
EtOAc	Ethyl actate
EtOH	Ethanol
FCPC	Fast Centrifugal Partition Chromatography
g-force	Gravity force
H_3PO_4	Phosphoric acid
HCl	Hydrochloric acid
HCOOH	Formic acid
HFBA	Heptafluorobutyric acid
HPCCC	High Performance Countercurrent Chromatography
HPLC	High Performance Liquid Chromatography
HSCCC	High Speed Countercurrent Chromatography
i.d.	Internal diameter
iso-PrOH	iso-propanol
JFAEC	Japanese Food Additives Expert Committee
K, K_D	Partition coefficient
KOH	Potassium hydroxide
LD_{50}	Lethal dose (amount of ingested substance that kill 50% of tested samples)
MeOH	Methanol

NaOH	Sodium hydroxide
NaCl	Sodium chloride
NADES	natural deep eutectic solvent systems
n-BuOH	n-butanol
n.d	not identified
$(NH_4)_2SO_4$sat	Saturated ammonium sulphate
NMR	Nuclear Magnetic Resonance
nm	Nanometer
No	Number
O. dillenii	*Opuntia stricta var dillenii*
O. ficus	*Opuntia ficus indica*
PFPA	Pentafluoropropionic acid
ppm	Part per million
Prep	Preparative
$R_{i,j}$	Resolution between two peaks i,j
rpm	Revolution per minute
S_f	Retension of stationary phase
SPSS	Statistical Package for the Social Sciences
TBMe	Tert-Butyl methyl ether
TFA	Trifluoroacetic acid
TFA-d_1	Trifluoroacetic acid –d_1
TLC	Thin Layer Chromatography
UV-VIS	Ultraviolet-Visible
V	Volume
v:v	Volume ration
V_C	Total column volume
V_S	Volume of stationary phase displaced in the equilibration of the system
V_{ext}	Volume of external connection in the CCC device
V_R	Elution volume of specific compound
V_{SF}	Volume of solvent front or volume of mobile phase
W	Width at base of the peak
λ	Wavelength in nm
δ	Chemical shift
Δ*m/z*	neutral loss

1. INTRODUCTION

Color is one of the most important attributes of food products next to flavor and texture, which are typical quality indicators affecting customer buying preferences activities.[1] Historically, colorant additives appeared in Ancient Egypt around 1500 B.C. and were substantially used to enhance the original colors of food, to standardize the batch's appearances and recover the color lost under processing.[2] These additives were derived from natural sources such as plants, flowers and minerals until late 1800s, when synthetic alternatives were discovered and became popular in industry because of their low price, high stability and possibility of mass production.[3] Nevertheless, the rapid growth of these 'petroleum oil originated dyes' not only disguises low quality foods but also causes a warning of detrimental health effects on children such as allergies, hyperactivity, sexual development, and colorants have been shown to bind to human serum albumin recently.[4,5,6,7] These controversial issues led to the establishment of numerous food legislations to regulate colorants being used in daily products. These regulations vary widely amongst countries. Only 7 artificial colorants are permitted in the USA compared to 17 in the EU. For instances, Patent Blue V (E131) and Green S (E142) are not approved in the U.S. but allowed in the EU.[2] That legislative pressure in combination with the consumer disapproval upon artificial colorants bring a trend towards natural pigments with a yearly regular growth of 5 - 10% in the global market of colorants compared with 3 - 5% of the counterparts. These natural derived substances are safer and healthier in human nutriton since they are produced through non-selective physical processes from plants. They are found in co-existence with other nutraceuticals, such as polyphenols, vitamin B, C, riboflavin, etc. which could supply extra health benefits to food products.

Several species namely saffron, paprika, turmeric, beet roots, and petals of various flowers are typical sources of pigments, such as porphyrins, carotenoids, anthocyanins and betalains. These pigments have distinct color hues from red to orange which are intensively used in foodstuffs and beverages. Amongst them, anthocyanins are the most potential pigment class due to its appearances in many varieties of fruits and vegetables. However, the vulnerabilities through hemiketal formation upon very low pH together with color fade by chalcone and quinonoidal base transformation are the major drawbacks of this type of pigments.[8,5] Carotenoids which are suited for coloring of fat-containing foods such as cheese, joghurts and cakes also show easy degradation by impact of light and oxygen. Betalains, on the other hands, are independent on a wide pH range from 3-7 and also soluble in aqueous foods and are widely used for milk products. The group of hydrophilic betalain pigments display antioxidative effects and abundantly occur in some common crops as *Beta vulgaris, Opuntia spp., Hylocereus spp.,* swiss

chards, *Amaranthus spp.*, etc. These plants supply other nutraceuticals such as flavonoid glycosides in addition to the pigments, which enhance the health-promoting properties. The cacti related sources have a high capacity to grow in arid/dry drastic areas making them a promising source for pigment and phytochemical extraction next to the ruminant feed supply.[9]

Nevertheless, betalains are highly instable upon environmental and processing conditions such as temperatures, light, oxygen, metal ions, etc. The high sensitivity combined with the less brilliant color hues and probably restricted occurrence in few plant families limit their mass production. Up to present, these pigments are mainly supplied from beet roots (*Beta vulgaris*) extract accompanied with undesired flavors such as geosmin and high nitrate concentrations, while purified compounds are rarely commercially available. Therefore, studies on improvement of pigment stability within food matrices and search for alternative betalains sources are necessary topics in food technology.[10,11,12] The advanced technology nowadays might open large potential for innovative research in exploring new colorant sources.[13,3]

The preparative all-liquid purification techniques of countercurrent chromatography (CCC) and centrifugal partition chromatography (CPC) have been used numerously in the large-scale separation of natural products. This separation technology is based on non-solid support chromatography and displays that no sample is irreversibly lost under proper operating condition. That makes this technique gentler for sensitive compounds than traditional/chemical separation methods and becomes the method of choice for pigment production. CCC and CPC have been reported effectively for complex mixtures to recover bioactive compounds and pigments such as anthocyanins, carotenoids, chlorophyll and betalains, resulting in compounds sufficiently purified for bioassay evaluation.

This work was initiated to develop a large-scale purification process for betalains applicable on varieties of betalain producing species, using the advanced CCC/CPC techniques. The CCC isolation of these pigments extracted from *Beta vulgaris*, *Opuntia spp*., *Hylocereus spp*., *Atriplex spp*. have been occasionally discussed before. In this study not only the pigments constitutions of these species were investigated, also their interesting phytochemical profiles were examined. The high polarity of betalains pigment causes difficulties in chromatographic purification, particularly the choices of suitable solvent systems for CCC. The uses of ion-pair forming systems containing perfluorinated carboxylic acids are crucial to enhance the distribution of pigments between the two phases. However, the afterward remaining acids make the isolated pigments not applicable for bioassay and food products. It is evident that replacement of these acids by food grade chemicals is essential in industrial production of natural colorants.[14]

On the other hand, pigments extracted from betalain fruits/vegetables contain a lot of water soluble plant metabolites next to polar pigments. These compounds need an adsorption process

or chromatography step for elimination before the CCC fractionation. Instead of using expensive synthetic resins such as Sephadex (LH20) or C18 reversed phase material, this work was aimed to use cheap and bio-degraded material, such as chitin from shrimp or crab, to develop a fast and simple pre-purification process applied for varieties of betalain plants.

Within this study, both CCC and CPC were used to fractionate the same batch of pigments to compare their separation powers. The reproducibility within a small CCC scale-up study from analytical to preparative scale was also investigated. Additionally, the thermal stability of *Opuntia* pigments during processing was also explored. Others valuable substances such as flavonoid-glycosides with sulphate substitution present in *Atriplex* extracts were also fractionated and isolated by CCC.

2. LITERATURE REVIEW

2.1 BETALAINS – THE VALUABLE NATURAL COLORANTS

2.1.1 Betalains in food industry

In food industry, anthocyanins are known as the most applied red/purple pigments, while carotenoids are used as yellow/ orange colorants. Anthocyanins, however, are instable under neutral and basic conditions, which limit their uses in several extremely low pH value food products; while carotenoids are not dissolvable well in water. Instead, betanins (betacyanins and betaxanthins) exhibit a wide spectrum of color hues from red/purple to yellow which is comparable to that of anthocyanins and carotenoids. Moreover, betalains showed higher color stability within a broad pH range from 3 to 7 and better water solubility compared to anthocyanin and carotenoids, respectively. These advantages make betalains attractive for certain applications. The uses of beet root betalains in food industry has been authorized by European Union under label E-162 as coloring additives for food, drugs and cosmetic products; and to date, there is a wide range of neutral and low-acidic foods such as milk products, desserts, candies and meat products that used E-162 as a coloring agent.[15,16]

Nevertheless, the world production of betalains is still underdeveloped since there is no simple purification methods without any degradation,[17] although an approximately 96.8 Gton betalains was potentially produced every year from red beet extract which accounted up to 99.99% of betalains production. The supplement counted only 10% of the natural colorants demand in 2009.[15] More efforts will be required to process and promote the application of betalains in the enlarged world colorant market, which is predicted to be prosper in the future.[3]

2.1.2 Betalainic plants and their health benefits

Betalains appear as a substitute for anthocyanins within few families of the order Caryophyllales.[16] These water soluble compounds occur in many parts of the plants, concentrate in flowers, fruits, roots and vegetative tissues. Their contribution to the stress regulation, nutritional transportation, tissues protection and plants propagation is in analogy to that of anthocyanins.[12] Up to now, red beet root (*Beta vulgaris spp., Chenopodiaceae*) is still the main betalainic source despite their undesirable flavors caused by geosmin, pyrazines, and the high nitrate content which suspected being associated with cancerogenous nitrosamines formation in human.[10] The uses of yellow beet pigments are not applicable due to the co-existences of phenolic compounds destructing the betaxanthins.[12] Numerous studies have been done to look for alternative commodities, from which 17 new betalain producing families had been found including the most promising *Cactaceae* family (*Opuntia spp*., *Hylocereus spp*., and *Mamillaria*

spp.). These results not only revealed a few valuable replacements, but also raised significant changes in the betalain perspectives as natural food colorants.[9] Several leafly vegetable have been also suggested as genus Amaranthus (*Amaranthaceae*), Swiss Chard (*Beta vulgaris L. cicla*) while the *Phytolacca americana L*. is no longer allowed to be used as food colorant due to their saponin and lectin contents.[18,12] The pigments are also reported in some ornamental plants as *Bougainvillea* bracts (*Nyctaginaceae*), *Gomphrena globose* flowers (*Gomphrena*), *Basella alba* fruits, *Suaeda salsa, Ullucus tuberosus, Chenopodium formosanum, Rivina humilis*.[19,15] Interestingly, betalains were found also in fungi or mushrooms such as fly agaric (*Amanita muscaria*), Hygrocybe, and Hygrophorus.[9] The colors of these betalainic plants depend not only on the pigment's chemistry, but also on edaphic conditions such as seasons, soils, irrigation, post-harvest processes, etc. The combination of these elements induced significant differences in color of each specie.[9,20]

Betalains health promoting benefits such as antioxidant, radical-scavenging, anti-inflammatory, antimicrobial, antiviral and anticancer properties have been approved. Their significant electron-donating capacity have been claimed to contribute in regulating oxidative reactions, inhibiting the degenerative diseases such as cancer or cardiopathies in human.[21,22] Stintzing and Carle (2004), then Henriette (2009) summarized the positive effects (*in vitro*) of betalainic species on skin and lung cancer, tumor inhibition, lipid oxidative damage reduction, radiation protection, etc.[10,12] Their antioxidant capacities were demonstrated to be more efficient than a derivative of vitamin E- Trolox, tocopherol, catechin and ascorbic acid. These bioactivities of betalains vary depending on the physicochemical conditions such as pH; e.g. the pH above 5.5 enhanced the antiradical activity of the pigments. The chemical structures of the pigment also showed a remarkable effect on its biological effects. [9,23,24]

2.1.3 Betalains classifications, structures and derivatives

Betalains are N-heterocyclic compounds which are classified into two subclasses: the red/purple betacyanins and yellow/orange betaxanthins based on their resonating double-bond structures. Betanin and indicaxanthin were the first betacyanin and betaxanthin being identified. They are also the most dominant pigments amongst 78 betalainic structures naturally occurring which have been reported recently.[25,26] These two pigments are abundantly present in red beet, *Opuntia spp*. and *Hylocereus spp*. The distinct colors of each betalainic species result from the varied ratios of betaxanthin and betacyanin contents.

Betacyanins and betaxanthins were wrongly considered as nitrogenous anthocyanins and flavocyanins for a long time until 1968, when the general term 'betalains' was suggested by Mabry and Dreiding;[27] they also found the chromic 1,7 -diazaheptamethin cation (Figure 2.1a)

responsible for the wide range hue of these compounds. This chromophore has bright yellow color and present within the structure of betalains precursor - betalamic acids (*4-(2-oxo-ethylidene)-1,2,3,4-tetrahydro-pyridine-2,6-dicarboxylic acid*). The conjugation of betalamic acid with variable proteinogenic/non-proteinogenic amino acids results in betaxanthins which display maximum absorption between λ 460 and 480 nm. Vulgaxanthin I appeares as one of the most abundant betaxanthins next to indicaxanthin (Figure 2.1d).[25] There are 18 new betaxanthins derivatives elucidated from sub family of *Chenopodiaceae* and *Cactaceae*; these new structures are also recorded as minor pigments in yellow beet.[28] Betacyanins are generated by the condensation of betalamic acid with the aromatic system dopa (*cyclo-L-(3,4-dihydroxy-phenylalanine)*) to form the unifying aglycone betanidin, which reveals particular maximum absorbance at λ 540 nm. Further characterizations of this aglycone with glycosylation and additional acylation create a great variety of derivatives (Figure 2.1b)

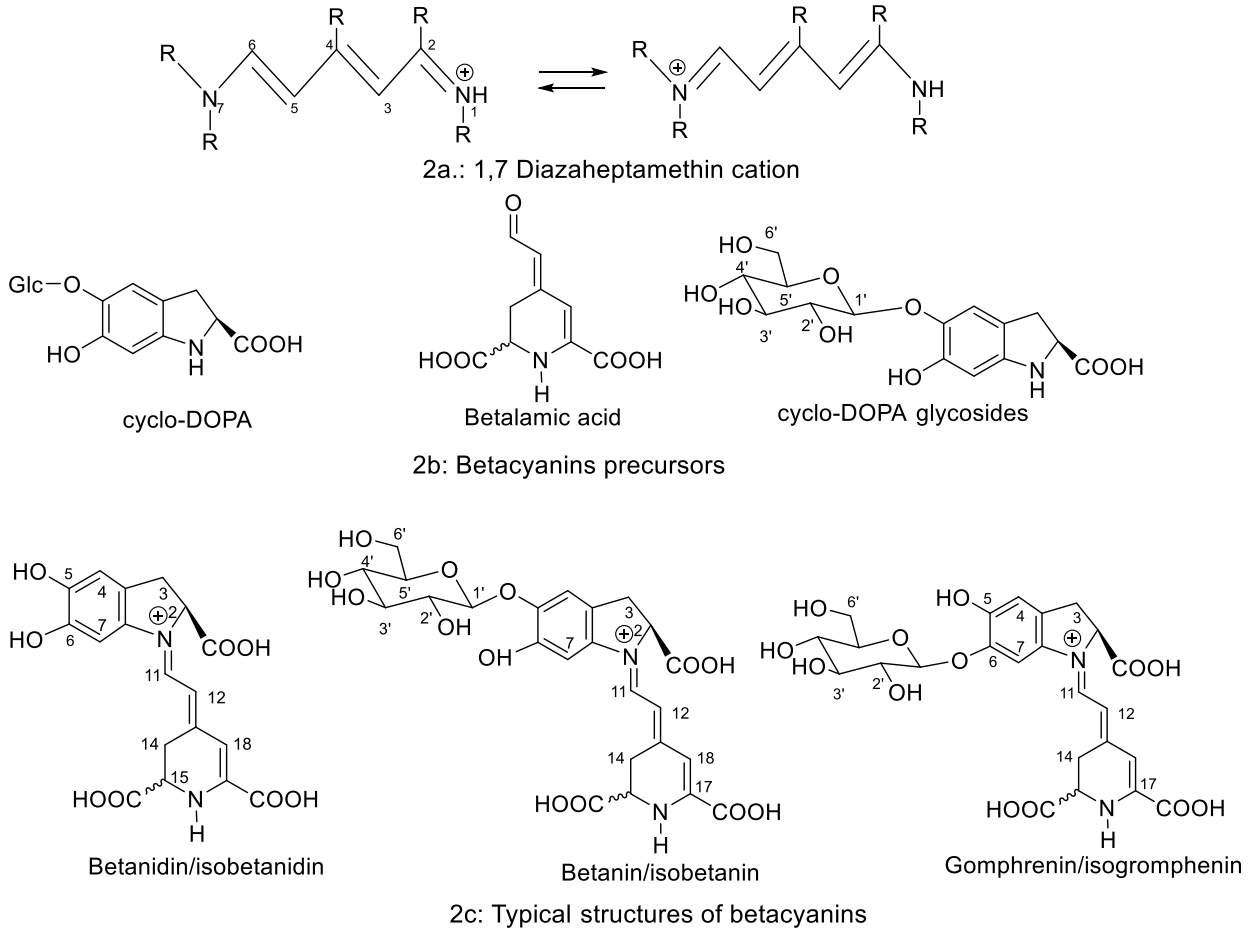
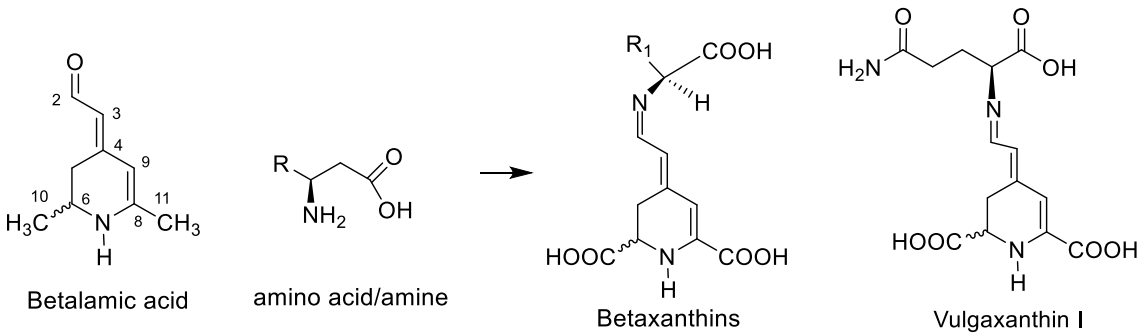


Figure 2-1: Betalain precursors and several typical pigment structures



2d: Betaxanthins formation and typical pigments

Figure 2-1 (cont): Betalain precursors and several typical pigment structures

The typical glycosidic substituents are monosaccharides namely glucoses, apiose and occasionally glucuronic acid, whereas the aliphatic acids or aromatic acid such as malonic, 3-hydroxy-3-methyl-glutaric, caffeic, p-coumaric, ferulic and sinapic acid are the principle acyl substituents.[26,8] Depending on the substitution position at C5 or C6, the corresponding structures are identified as betanin or gomphrenin (Figure 2.1c). Betacyanins display two stereogenic centers at C2 and C15; the formers are not resolvable by recent techniques while the later are more described. These C15 stereoisomers are always co-existing, in which the 15*S* was overwhelming the 15*R* counterpart.[27] Figure 2.1 illustrates the chemical structures of the precursors and several typical betalains.

The substituent structures cause variation in the chromic absorption of both betacyanins and betaxanthins. Stintzing et al. (2008) summarized the effects of different structures on betalains maxima absorption; e.g. the 6-O- glycosylation and aromatic acylation led to the bathochromic shift λ of 5 to 6 nm compared to the 5-O- and aliphatic substituents in betacyanin. Whereas the increase of two $–CH_2$ units in the amino chain length or the decarboxylation results in the 7 nm and 10 nm hypsochromic shift in betaxanthins, respectively.[8,29] These structural differences not only influence the chromatic absorption, but also the biological activities. Generally, the glycosylation and acylation significantly change betacyanins bioactivity; i.e., betanidin shows highest antioxidant capacity against peroxyl radicals and nitric oxide because of its available free 5,6-dihydroxyl catechol moiety, which is not present in its glycosides. The 6-O glycosylation exploits better bioactivities than its 5-O compartments, and acylation also enhances these abilities. On the other hand, the increased number of hydroxyl and imino groups improves radical scavenging properties of betaxanthins.[12,30]

2.1.4 Stability of betalains

The pigment stabilities depend on many specific factors namely pH, temperature, light, oxidation, antioxidants, enzymatic reactions, chelating agents.[12] In term of extrinsic parameters, betalains are found more resistant to acids than anthocyanins, and display a broad desirable pH range from 3 to 7. Within this pH range, a long-term treatment at high temperature are the most detrimental conditions for betacyanins causing degradation, namely isomerization, decarboxylation, dehydrogenation and hydrolytic cleavages. These reactions releases a variety of corresponding degraded products including isobetacyanin, mono-/ bi-/ and tri-decarboxy-betacyanins, dehydrogenated betacyanins or neo-derivatives, the acyl moiety together with the precursors betalamics acids and cyclo-dopa 5-O-glucoside.[25] The decarboxylation process at C2, C15 and C17 generates series of compounds namely 17-decarboxy-betacyanins/15-decarboxy-betacyanins; 2,17-bi-decarboxy-betacyanin and 2,15,17-tri- decarboxy-betacyanins, respectively. Degradation of betacyanins has a large influence on their light absorption. For examples, the losses of carboxyl groups at position 2 and 15 do not cause bathochromic shifts, whereas the 17 cleavage makes a significant reduction in λ of 30 nm and color change from purple to red/orange. Meanwhile, the betacyanins dehydrogenation occurs at C14–C15 which is characterized by a maximum absorption λ around 470 nm similar to that of betaxanthins.[8] These neo-derivatives are considered as betacyanins' thermal indicators because their yellowish color faded from original red/purple.

When pH is out of the optimized range, these degradation reactions are strongly provoked. For instance, betacyanins isomerization is not only enhanced by high temperature, the alkali and acidic environment also show correspondences. This epimerization occurs at C15 resulting in no color difference but the changes in 15*S* and 15*R* configurations ratios, in which the later is promoted.[20,31] Under processing, these degraded compounds in combination with the food components make a varied spectrum from reductive red to light yellow and brown. Noticeably, the stability of betacyanins appeared in correlation with the food compositions;[32] which are suspected to be protective matrix for the pigments during processing compared to the separated pigments.[26]

Betaxanthins, on the other hand, generally display less resistances than betacyanins under both normal and high temperatures; e.g. they are more susceptible to hydrolytic cleavages and decarboxylation at C11 and C13 during thermal treatment. The heat treatment generates from indicaxanthin the degraded compounds including their precursor cleavages of betalamic acids and amino acids, and the stereoisomers at C11 as well.[8] Additionally, weak acidic pH shows positive effect on reducing thermal destruction of betalains; several acids such as ascorbic acid and citric acid are used as pigments stabilization in food industry. For instance, 0.1% of ascorbic

acid could extend the half-lifetime of both betaxanthins and betacyanins. Nevertheless, these activities are less pronounced under light exposure and exhibit variances towards different betacyanin structures.[33] The ambient atmosphere containing high levels of oxygen and/or other radicals probably enhance these degradations in contrast to nitrogen atmosphere. von Elbe et al. (1974) reported the two folds of betanin decomposition under simultaneous presence of oxygen and light; these reactions were identical to those of metal cations such as Fe^{2+}, Fe^{3+}, Sn^{2+}, Al^{3+}, Cr^{3+}, Cu^{2+}.[34,35,36] Besides, enzymes as β-glucosidases, peroxidases and polyphenoloxidases also promote the degradation, therefore deactivation steps are required in food processing. Noteworthy, the enzymatic susceptibility of these pigments is dependent of structural features; and betacyanins are more sensitive to peroxidases while betaxanthins are unstable under hydrogen peroxide oxidation.[37] In general, all these environmental elements in combination cause distinct contributions to the instability of betalains.[25]

Next to these external conditions, the internal factors such as structures, concentration, and matrices' constituents also show principle responses to pigment stabilities. The effects of chemical structures are of high interest and are well investigated, particularly on betacyanins. Generally, the glycosylation is reported to stabilize the pigments upon oxidative species in comparison to its aglycone. However, that oxidation–reduction potential is not enhanced more by further glycosylation. Whereas the positive effects of both aliphatic and aromatic acylations on stabilization are also illustrated; in which the esterification at 6-*O*-position are more effective than that of the 5-*O*-compartment. These aliphatic acylated derivatives protect the aldimine bond of pigments by the intramolecular associations with betanidins, and similar to tracking effects of aromatic moieties.[38] In addition, the pigment consistencies corresponding to their concentration have also described.[39,25] Herbach et al. (2004) introduced the degradation pathways of red beet betanin under thermal treatment and their appearance color;[40] lately Wybraniec et al. (2013) added the general degradation scheme of betanin derivatives by oxidative radicals.[37] Interestingly, the regeneration of betanin by condensation of their hydrolysis precursor are induced. Betalamic acid and cyclo-dopa incubated under low temperature (below 10^0C) and acidic pH (5.0) are reported to re-condensate, which open the chances for betacyanins regeneration.[41,42]

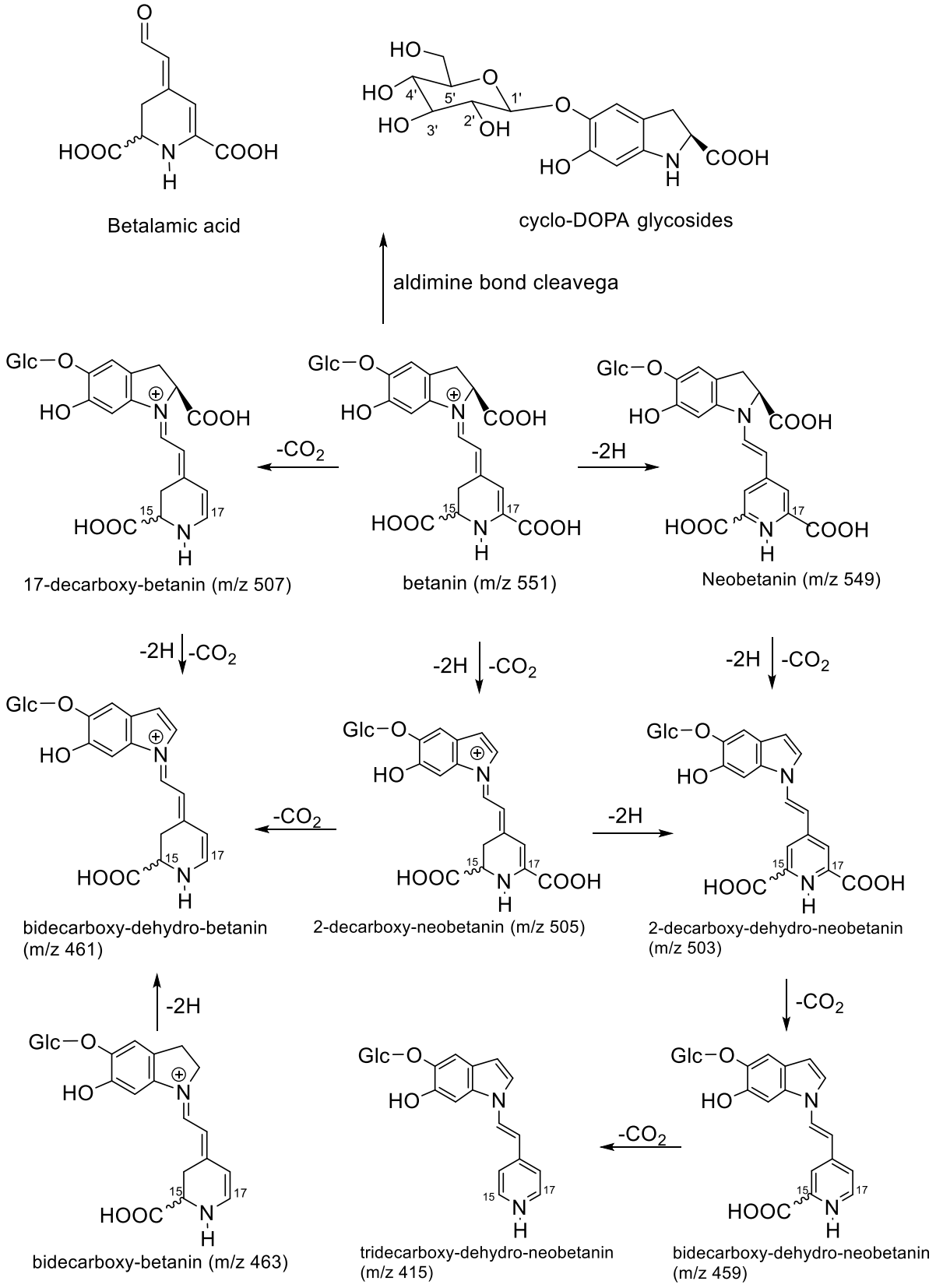


Figure 2-2: Degradation pathways of betalains (modified from Wybraniec et al., 2013, Herbach et al., 2004)[37,40]

2.1.5 Betalains purification and analysis

2.1.5.1 Extraction and enrichment

Naturally, pigments are more resistant within the intracellular compartments consisting of polyphenolic compounds which act as protective environment. The processing might breakdown these interaction between the pigment molecules and their matrix, and make them more vulnerable. Water is typically used for betalains extraction because of their high solubility; many other polar solvents are found to assist the extraction including methanol, ethanol, acetone and ethylene glycol, etc. Although the alcoholic solutions claimed to enhance the instability, the use of ethanol is favorable since the increasing attempt of using food grade ingredients in food industry. For instance, the mixture of methanol or ethanol with water (20–50%) have been applied to maximize the extraction yields, and the addition of small amount of acid is known to cut down the polyphenoloxidase enzymatic reactions.[26,43] There were also some novel extraction methods introduced recently such as fermentation, pulsed electric field treatments and supercritical fluid extraction, which are pronounced effectively supporting the betalains extraction and being environmental friendly in the processes.[44,45]

Many synthetic resins have high adsorption capacity to plant extracts that their applications on pigment enrichment become more important in the food and pharmaceutical industry.[46] On lab-scale, the concentration of pigments has been done with some common solid phases such as silica gel, end-capping C18, Sephadex (LH20) and organic cation-exchanger (XAD-7, XAD-17). However, these resins have been claimed promoting rapid degradation of labile pigments (betalains) during desorption by solvents. The novel aqueous two phase systems (ATPS) consisting of polyethylene glycol and ammonium sulphate is introduced for purification of red beet juice pigments lately. This method not only releases pigments from high dose of the beet sugars but also fractionates betaxanthins and betacyanins. Nevertheless, these polyethylene glycols make strong interaction with the pigments and require release by solvents, such as chloroform, which is not considered as a food grade chemical.[47]

2.1.5.2 Betalains analysis

In food industry, the color changes during treatments are important for quality control and understanding of customer’s selection. The color of betalainic products is defined by total content of betacyanin and betaxanthin pigments which are present in varied ratios depending on commodities and process-induced alterations. There are typical degradation compounds which are indicators for distinct processing steps; e.g. neo- and decarboxy- derivatives are indicators for thermal treatments, increased generation of betalamic acid is raised by long term heating and

storage, whereas insufficient enzymatic activation lead to glycosides hydrolytic cleavage of glycoside bonds and release of sugar units.[26]

Historically, UV-Vis spectrophotometry is the most straightforward method for betalains quantification despite the claims that pigments are underestimated and information about structural alteration are not displayed.[48] Meanwhile, others analysis approaches have been developed to apply for pigments from single commodity such as *Beta vulgaris* or *Amaranthus tricolor*. These analysis were then found to be not applicable to other cultivars; e.g. the quantification method suggested by Nilsson in 1970 was successful on red beet pigments but not on cacti species.[49] The introduction of HPLC and then combined with spectrophotometer become the methods of choice for betalains phytochemical characterization.[48,50] However, the unavailability of the references causes difficulties in identification of new pigments, particularly in betacyanin derivatives. Lately, UV-Vis together with LC-MS could display the masses of fragmented ions which allow the structural elucidation of degradation products. Recently, 1D (^{1}H, ^{13}C) and 2D-NMR (COSY, HSQC, HMBC…) are taken part in the approach resulting in complete structural clarification of similar molecular weight compounds. The latest technique has been applied effectively to explore pigments from red beets, *Opuntia spp.* and pitahaya fruits (*Hylocereus*), swiss chards, and their alterations under processing.[26,51,52,53,54]

2.2 COUNTERCURRENT CHROMATOGRAPHY – A PREPARATIVE ISOLATION METHOD APPLIED FOR PLANT DERIVED PHYTOCHEMICALS

2.2.1 Overview

Countercurrent chromatography (CCC) is an all-liquid chromatographic technique relying on the partition distribution of samples within biphasic immiscible solvent systems. In principle, this approach consists of multiples continuous liquid-liquid extraction processes, which enable high loading capacities from milligram (analytical) to kilogram (industrial) scales. It allows full recovery of loaded samples by eliminating the irreversible adsorption and degradation occurring on the solid-support in comparison to preparative HPLC. CCC offers more than one operation modus that is flexible for a wide range of sample polarities and solvent systems. In addition, the prediction possibility for the expected elution volumes, and possibility to couple with online/offline detection methods, such as UV-Vis and ESI/ACPI-MS/MS, make CCC more advantageous than other purification approaches.[55] This liquid-liquid chromatography has been applied numerously in larger scale fractionation of proteins, nanoparticles and many highly fragile plant derived products such as polyphenols, glycosides, terpenes, steroid derivatives, etc.[14,56] DAD/prep-HPLC and HPLC/MS-MS are typical techniques used in pigments

purification. They have induced complete identification and colorimetric measurements of colorant phytochemistry from many food systems. However, the insufficient efficiency together with expensive experimental set-up keep those methods more applicable in lab-scale instead of commercial production. Meanwhile, the recent growth of natural colorants market gains a demand on the large-scale purification approaches with affordable operation. CCC seems to be the alternatives in which costly resins are replaced by versatile solvent systems involving very gentle operation conditions. These conditions are critical for highly susceptible pigments to reduce the denaturation compared to the solid active-surfaces adsorption. These advantages make CCC numerously used to investigate a varieties of natural products including distinct polarity pigments such as carotenoids, chlorophyll, anthocyanins, and betalains.[14]

2.2.2 Principles of CCC

2.2.2.1 Retention of stationary phase

The sufficient retention of liquid stationary phase in the coil column together with the equal partition of samples between two immiscible phases are the key parameters of CCC methodology. Under processing, the stationary phase is kept inside the instrument by the centrifugal force fields while the mobile phase is pushed through. The stationary phase initially filled into the coil column is replaced by the mobile phase until the hydrodynamic equilibrium is achieved and when only mobile phase will be released. That stage allows thousands of mixing/demixing cycles along the tubling length, where the repetitive partitioning of solutes between two phases happens continuously. The centrifugal force fields are created via a planetary motion in combination with the multilayer coil configuration displayed in Figure 2.3.

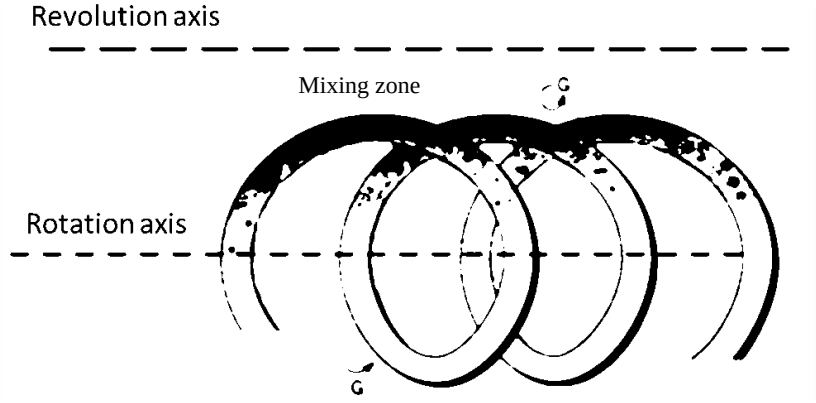


Figure 2-3: Planetary motion in CCC apparatus. (Berthod et al., 2009)[57]

The volume of stationary phase hold in the coil at equilibrium is called retention and represented by stationary phase retention factor S_f, which is unique for every single separation and solvent system. S_f is calculated as the percentage of stationary phase retained in the system (Eq. 2.1):

$$S_f = \frac{V_S}{V_C} * 100 \quad \text{(Eq. 2.1)}$$

V_S and V_C are the stationary phase volume retained and total coil volume, respectively. In practice, the extra coil volume or "dead" volume comprising of connecting lines should be taken into account, especially in the analytical-scale separation.[58]

The sufficient S_f (above 50%) is required for a good separation because it is proportional to the separation capacity and the amount of sample injected according to Ito (2005) and Sutherland et al. (1987).[59,60] The S_f values are fluctuating depended on the natures of the solvent system, the operation flow rate, the revolution speed and the machine's configuration (e.g. inner coil diameter and revolutional radius, etc.). Many studies had been done to investigate the effects of each parameter on S_f; showing the negatively relation between it with increased flow rate whereas the bore tubing sizes display positively correlation. These parameters in combination strongly make S_f changes and might reach above 90%. These studies suggest the possibility for CCC performance optimization.[61]

2.2.2.2 The two phase solvent systems

The selection of appropriate two phase solvent systems for a crude extract could be one of the most challenging and time consuming aspect of a CCC separation. Generally, the systems should satisfy some prerequisites namely samples dissolvability and stability, no degradation induced by solvents and equal volume ratio between two phases for economic and environmental reasons.[59] Either phase of these two immiscible liquids can be the mobile phase and/or stationary phase which so called upper phase (lighter phase) and lower phase (heavier phase or denser phase). During operation, the phases move towards the two opposite ends of the coil column named 'head'-end and 'tail'-end according to the 'Archimedean Screw Force' effect (Figure 2-4a). These two ends are exchangeable depending on rotation direction (counter clockwise and clockwise), in which the heavier phase occupies the tail-end while the lighter moves to the head-end (Figure 2-4b). These revolution flexibilities offer many elution modes to the CCC approaches.

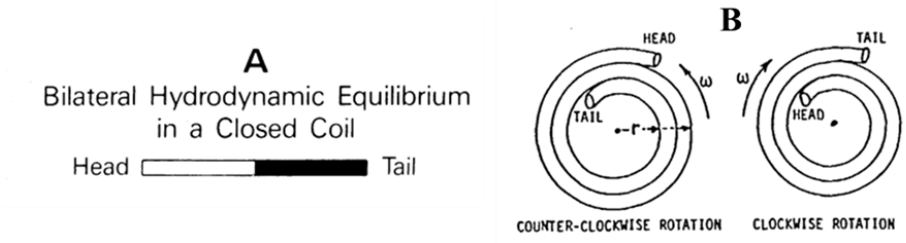


Figure 2-4: Relation of 'head' and 'tail' ends in a closed equilibrium coil (A) and under two rotation directions (B). Modified from Ito (2005) and Ito et al. (1986) [59,62]

The constitutions of the systems are critical for a successful separation and should be studied carefully. There was numerous work done on the correlation between composition and physical properties of solvent systems exhibiting different polarities, and the application of these systems in CCC separation in practices. These studies suggested that the differences in density, viscosity and interfacial tension between the two phases are important to ensure the short settling time, which leads to fast equilibrium. In a CCC operation, the retention of over 50% represented by a fast settling time is encouraged. The systems with long settling times (more than 30 seconds) could be adjusted by electrolytes such as acid or salts; these supplements could enhance the density differences between two phases and also eliminate the emulsification. However, the addition of highly concentrated salts (saturated salt) might induce the risk of precipitation, and requires extra step to desalt the separated fractions later. This makes the acidification preferable in practice.[62]

The solvent systems could be classified into three groups according to their polarities: the hydrophobic, the medium and hydrophilic groups, which indicate distinct hydrodynamic behaviors under planetary movement.[62] The first two groups have been applied effectively for CCC separation of non- or less- polar compounds, whereas the application of the last group for polar solutes such as proteins and polysaccharides are still limited. Recently, the novel aqueous two phase systems (ATPS) made of polyethylene glycol and saturated salt solutions, and the ionic liquid systems have been used effectively in the separation of polysaccharides, polypeptides and proteins.[56]

These highly polar solvent systems consisting of heavy alcohols (e.g. n-butanol) display a long settling time spontaneously resulting in insufficient retention. In addition, these hydrophilic systems also exhibit emulsification tendency which not only hamper the equilibrium, but also cause flushing out (or carry out) of stationary phase during processing.

Recently, the natural deep eutectic solvent systems (NADES) consisting of solids associating with each other to form viscous liquid have been applied into CCC. This mixture requires a hydrogen-bond donor and a hydrogen-bond acceptor (such as choline chloride and urea, respectively) which hydrogen bonding interactions reduce the mixture's melting point to lower value than that of initial ingredients. The natural source and easy accessibilities make these eutectic systems prospective approach for CCC in the aspects of ecology and toxicology in comparison to the organic solvent systems; whereas the physical properties (density, viscosity) are comparable.[63]

2.2.2.3 Partition coefficient K_D and elution modes

There are various existing names for partition coefficient K_D in older and recent literature, as there is no guiding agreement on a unique IUPAC nomenclature. The K_D (or K) was also named as partition -ratio, -value, -factor or distribution factor. This value is the ratio of solutes concentration present in each phase, and could be calculated by equation 2.2, in which C_U and C_L are the concentration of the compound in upper and lower phase (when lower phase is mobile phase):

$$K = \frac{C_U}{C_L} \qquad \text{(Eq. 2.2)}$$

The K values are measured through a partition study using TLC, HPLC or LC-ESI-MS. These values are of interest for CCC as they allow in certain cases the prediction of elution volumes V_R of the specific analytes according to the equation suggested by Ito (2005):

$$V_R = V_{SF} + K^* (V_C - V_{SF}) = V_{SF} + K * V_S \quad \text{(Eq. 2.3)}$$

in which V_{SF} is solvent front or the volume of mobile phase passing through the coil during equilibrium, and V_S is the volume of stationary phase. The compounds with high K value indicate a strong affinity to upper phase combined with large elution volumes. Whereas the small K values occur when the analytes are enriched in the lower phase because of very high polarity. For a good separation, the preferred K range is 0.5 – 1.5; it is important for solvents and elution modes selection. Ito (2005) had introduced the procedures to search for appropriate solvent systems including the K_D evaluation. The analytes with distinct distribution in a single phase could be modified by 'hypophilization' such as perfluoro carboxylic acids or acetic acid. This has been applied in separation of water soluble pigments, e.g. perfluoroacetic acids have been used numerously to enhance the distribution of betalains and the retention of stationary phase.[59]

The elution mode is also an important parameter because it could affect the separation efficiency and shorten the operating time. There are two typical elution modes of normal phase (*'tail to head'*) and reverse phase ('*head to tail*') based on the choice of mobile phase being upper or lower phase. The development in CCC methodology and instrumentation lead to the breakthrough in elution modes applicable for such complex compounds.[64] Figure 2-5 illustrates the '*head to tail*' elution mode in combination with elution-extrusion approach in correlation to K_D values of compounds.

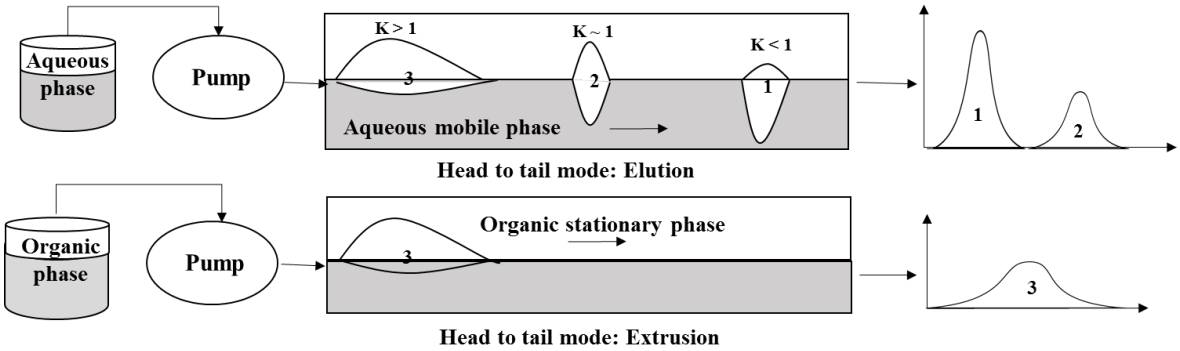


Figure 2-5: Overview of '*head to tail*' elution mode. Modified from Berthod et al., 2009[57]

2.2.3 Instrumentation

The first CCC description was introduced in the 1960s by Ito, since then many developments in geometry of the coil column had been made until the modern machines are commercially available nowadays. There are two categories relied on the hydrostatic or hydrodynamic way that the gravitational forces keep the stationary phase in the columns: centrifugal partition chromatography (CPC) and coil planet centrifuge (or high speed CCC (HSCCC)), respectively. Briefly, the CPC operates under a constant centrifugal force field implied by a single-axis rotation, while the HSCCC bases on a variable gravity force field produced by a multi-axis rotation (Figure 2.6).[65]

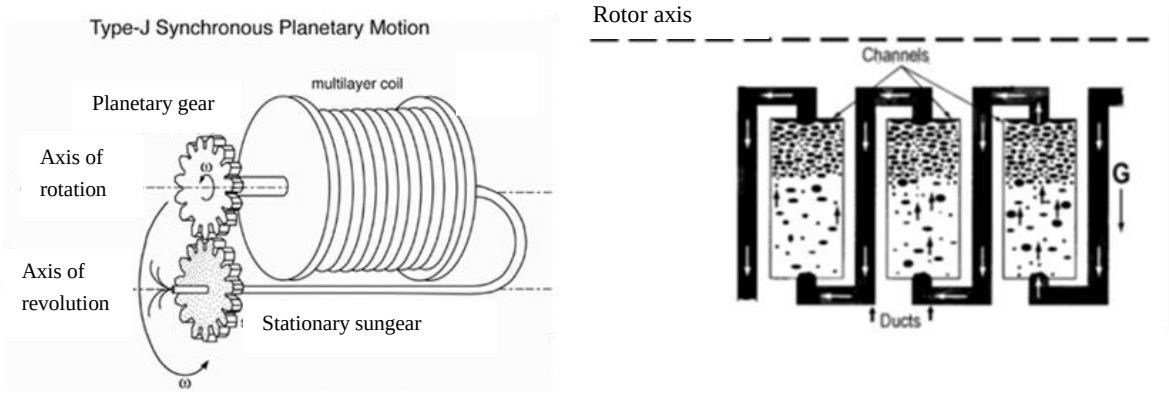


Figure 2-6: Hydrodynamic column (planetary motion, left) (Ito, 2005)[59] **and hydrostatic column (CPC, right) (Berthod et al., 2009)**[57]

In HSCCC, the chromatographic column is made by a long Teflon tube wrapped in multilayers to form a bobbin, which could be assembled singly accompanying with a counter balance or connected in series of up to four compartments to balance themselves. The system spins synchronously around its own "planetary" axis (central axis) and the "solar" axis (centrifugal axis) located in the center. The machines have been improved gradually by Ito and colleagues until the latter equipments namely J-type centrifuges with planetary motion were introduced and

commonly used recently. The new design has the rotating parts consisting of rotors, planetary gear and sun gear, flying lead connectors, motors, and speed regulators that allows both movements operating at same angular velocity and direction (Figure 2-6, left).[57] The generated centrifugal force displays more complex heterogeneous motion within the coil columns resulting in better retention of stationary phase and anti-twist tubing mechanism compared to the old versions.[66,67] A typical bobbin could handle up to 350 loops enabling thousands simultaneous mixing and settling zones during rotation. These zones travel synchronously toward "head" end of the column, in which the mixings happen close to the spinning center where the force fields are weak, while the settlings take place furthest by the stronger forces (Figure 2-7, left). The tubing inner diameter is around 1.6 - 2.6 mm with the maximum length of 160 m; they could be connected in series for higher chromatography volumes and resolution that enables more sample to be loaded.[14] This mechanism allows the analytes taking part in 1000 mixing/settling rounds per minute when the rotation speed is 1000 rpm, and brings about better isolation capacity for a varieties of natural products. The recent HSCCC generation, so called high performance CCC (HPCCC), has upgraded centrifugal force field up to 240*g or 1600 rpm, and has been proved to retain better stationary phase. This robust apparatus is capable with higher flow rates than the old version, and could make the separation reproducible within shorter operating time. The separation time is also a very important parameter for such a preparative approach applied for the labile natural pigments. Figure 2-7 (right) introduces the new HPCCC apparatus from Dynamic Extractions.

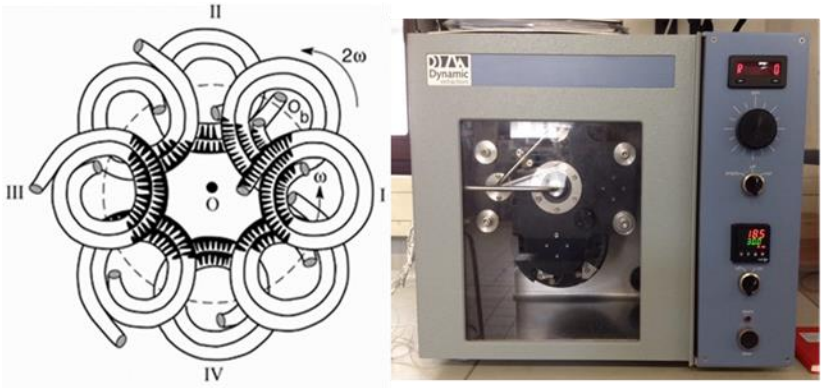


Figure 2-7: The distribution of mixing (dashed regions)/settling (white regions) zones within planetary motion (Ito, 2005) and HPCCC apparatus (model: spectrum with twin coil system, total preparative coil volume 125ml-Dynamic Extractions, Gwent, UK)

2.2.3.1 CPC

The hydrostatic CPC configuration is made of an adapted rotor that spins around its own central axis and comprises of arranged rotary seal joints connecting the rotating system with the static compartment. The rotor is assembled by a round cartridge constructed with multilayer-connected

staked plates, where hundreds of channels and ducts are circularly engraved and linked in series (Figure 2-8). During operation, the centrifugal force keeps stationary phase retained in the CPC rotor while the mobile phase is pumped through the ducts in to the channels. The flow of mobile phase forms a stream of droplets which is surrounded by the stationary phase inside the channels, where the mixing and separating steps happen. The mobile phase is continuously crossing the ducts to the next channels and the next plates before eluting out.[66]

Depending on physicochemistry of the biphasic solvents and natures of the analyses, either "ascending" or "descending" elution mode could be used for a proper separation. In the descending mode, lower phase works as mobile phase which is pushed from the top in the same direction with centrifugal force filed inside the rotor. While the ascending mode operates with the lighter-mobile-phase pumped from the bottom and opposite way of rotation direction. Ascending and descending modes are defining the direction and operation mode such as "*head to tail*" or "*tail to head*" modes in CCC, respectively. These modes are designed to maximize the contact between two phases, and suitable for solutes displaying high affinity to the either lower phase or upper phase accordingly. The modern fast CPC (FCPC) allows the high speed performance up to 1200–3000 rpm, which enhances the retention of stationary phase at superior flow rate of 5-20 ml/min.[68] Comparable to CCC, the risk of stationary phase stripping under raised pressure and high flow rates might flush out continuously the micro-droplets. The accumulation of pressure occasionally happens during operation depending on revolution speed, flow rates and nature of solvent systems (viscosity, density) and the channels spacial configurations as size and numbers.[69,70]

The CPC apparatus has been proven to work effectively with highly polar solvent systems (e.g., binary butanol/water system, ATPS), and is applicable for superior hydrophilic compounds such as proteins and polypeptides. The high fractionation resolution together with scale-up ability make CPC competitive to CCC in the field of natural product on industrial scale purification recently.[71] Noticeable, there is a raise to use novel solvent systems such as deep eutectic solvent (DES) in CPC approach to separate the highly hydrophobic and/or hydrophilic plant originated compounds such as tocopherols.[63,72] Figure 2.8 introduces in detail the complete configuration of the CPC.

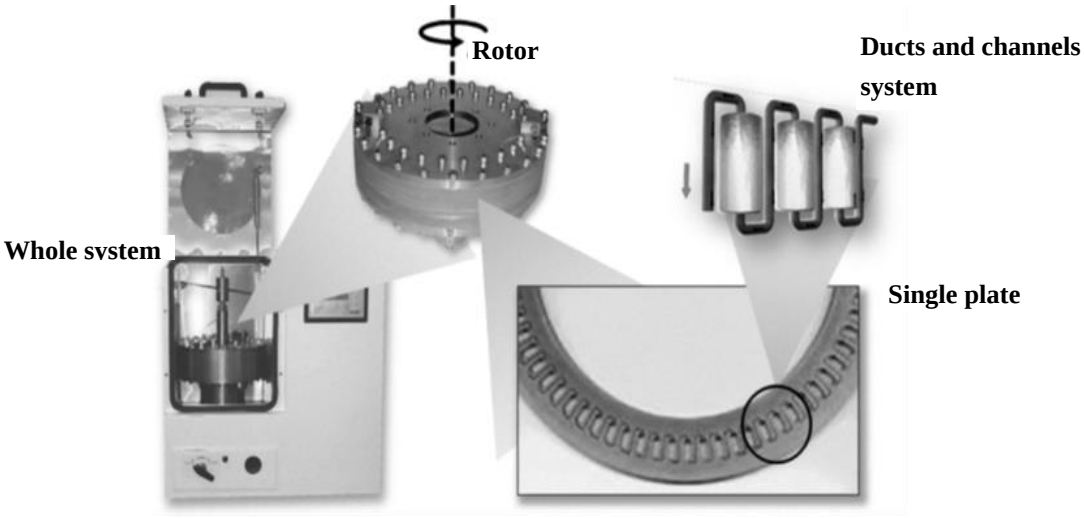


Figure 2-8: The CPC instrumentation in details (Minguillón, 2012)[66]

2.2.4 Summary of CCC applications on betalains

Generally, CCC/CPC is one of the most powerful large-scale purification methods in research, especially for fractionation of bioactive natural compounds occurring in very low concentration within complex crude plant extracts. This approach has been applied numerously on investigating natural pigments, particularly the betalains from a varieties of fruits and vegetable such as red beets, *Opuntia spp*., pitahaya, *Iresine lindenii Van Houtte*, etc.[45,73,74] Because of the extreme hydrophilicity of these pigments, it is difficult and time consuming in searching for suitable solvent systems applied in CCC. Historically, the salty systems of ethanol/ acetonitrile/ saturated ammonium sulphate/ water were used in the separation of betalains on HSCCC.[75] Later, the modified salty system introduced which is capable to isolate the mixture of red beet degradation products consisting of C17, C2 decarboxy-betanin, 2,17-bidecarboxy-betanin, neobetanin and betanin.[76] However, the highly salted solvents combined with CCC resulted in isolated fractions that required extra step to de-salt before bioassay could be performed. Recently, a new series of solvent systems modified by perfluorinated carboxylic acid have been introduced together with their application on many betalains producing plants. The pigment partition coefficients were enhanced by low concentration of acid (up to 1% depending on type of acid and pigments configuration). The acids strongly shift the distribution of pigments from the lower phases to the organic phase similar to the effects of the initial salty systems. Up to now, several acids have been found supportive for betalains separation including trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA). These ion-pair reagents not only change the physicochemistry of the solvent (pH, viscosity, density...) but also increase the lipophilicity of the betalains' diazaheptamethinium structures.[77,73]

Kucab et al. (2016) summarized the application of HSCCC and HPCCC on betalains separation and the applied solvent systems, which were classified into three categories: the system of TBMe-n-BuOH-ACN-water modified by ion-pair perfluorinated carboxylic acids, systems of BuOH-EtOH-NaCl-water-H_3PO_4 and the saturated $(NH_4)_2SO_4$ containing systems.[78] The first group has gained more interests recently because of its high efficiency in studying many pigment derivatives. For instance, these systems were capable to isolate betanin and its epimers in *Phytolacca americana* (*Phytolaccaceae*), the high molecular weight acyl-oligosaccharide betacyanins from *Bougainvillea* bracts (*Nyctaginaceae*), the betacyanins and betaxanthins in ripe *Cactaceae* fruits (*Hylocereus polyrhizus, Opuntia ficus*), betalains and their processed alterations in *Beta vulgaris L* juice (*Chenopodiaceae*), amaranthine, betanin and iresinin I from *Iresine lindenii* and so on.[45,73,79,80,74] Recently, the sinapoyl-gomphrenin I from *Gomphrena globosa L.* was purified successfully by the modified system of TBMe-n-BuOH-MeOH-water-HFBA;[81] or the amaranthines, betanins and acylated amaranthine types from *Celosia spicata (Thouars) Spreng* were also party fractionated by TBMe-n-BuOH-ACN-water-HFBA and EtOH-ACN-1-PrOH-$(NH_4)_2SO_4$sat-water.[82] Table 2.1 summaries the CCC methodology and solvent systems used in isolating betalains pigments from some betalainic varieties.

Table 2-1: Summary of betalainic varieties purified by CCC

Betalainic varieties	Principle betalains deravatives separated	Solvent systems (v/v/v/v)	CCC Apparatus and operation mode	Ref
Concentrated red beet juice	Betanin and isobetanin	EtOH-ACN-$(NH_4)_2SO_4$sat-water (1/0.5/1.2/1)	HSCCC-1000-PharmaTech Research-Cooperation (Baltimore, Maryland, USA) 850 ml; *Tail to head*	[75]
Thermal treated *Beta vulgaris*	betanin and its degradation products of decarboxy derivatives and neobetanin	n-PrOH-ACN-$(NH_4)_2SO_4$sat - water(1/0.5/1.2/1) EtOH-ACN-n-PrOH-$(NH_4)_2SO_4$sat - water(0.5/0.5/0.5/1.2/1) EtOH-n-BuOH-ACN-$(NH_4)_2SO_4$sat -water (0.5/0.5/0.5/1.2/1)	HPCCC — Dynamic Extractions Ltd., UK 18.4 ml; *Tail to head*	[76]
Processed red beet roots	betanin and its degradation products of decarboxy derivatives and neobetanin	n-BuOH-EtOH-NaCl-water-H_3PO_4 (1300:200–2000/1300/700/2.5–10)	HPCCC (Dynamic Extractions, Gwent, UK) 70.5 ml; *Tail to head*	[83]
Processed red beet roots	betanin and its degradation products of decarboxy derivatives and neobetanin	Highly polar solvent systems with salt Ion-pair solvent systems (perfluoro carboxylic acid)	HPCCC (Dynamic Extractions, Gwent, UK) 143.5 ml; *Tail to head* (salt systems); *Head to tail* (acid systems)	[84]
Gomphrena globosa L. flowers	amaranthin and decarboxy amaranthin; betanin gomphrenin I	$(NH_4)_2SO_4$sat-EtOH (2/1); EtOH-ACN-n-PrOH-$(NH_4)_2SO_4$sat–water(0.5/0.5/0.5/1.2/1.0); EtOH-ACN-$(NH_4)_2SO_4$sat-water (1.0/0.5/1.2/1.0)	AECS QuikPrep (London, UK) 121ml; *Tail to head* /*Head to tail*	[78]
Gomphrena globosa L. flowers	gomphrenins I, II and III, sinapoyl-gomphrenin I, cis-isomers of	TBMe- n-BuOH-ACN- water (2/2/1/5/0.7% and 1.0% HFBA) TBMe-n-BuOH-MeOH-water (2/2/1/5/0.7% and 1.0% HFBA)	AECS QuikPrep (London, UK) *Head to tail*	[81]

	gomphrenins II and III			
Iresine lindenii Van Houtte	amaranthin, betanin, iresinin I, feruloylated and sinapoylated betacyanins and hylocerenin	TBMe-n-BuOH-ACN-water (1/3/1/5/0.7% HFBA)	HSCCC model CCC-1000 (Pharma-Tech. Res. Corp., USA) 850 mL *Head to tail*	[74]
Atriplex hortensis var. rubra	celosianin II, betanin and amaranthin	TBMe-n-BuOH-ACN-water (1/3/1/5/0.7% HFBA)	HSCCC model CCC-1000 (Pharma-Tech. Res. Corp., USA) 850 mL; *Head to tail*	[85]
Bougainvillea glabra betacyanins	acyl-oligosaccharide linked betacyanins	TBMe-n-BuOH-ACN-water (2/2/1/5/0.7%TFA)	HSCCC model CCC 1000 (Pharma-Tech Research Corp., Baltimore, MD, USA) 850 mL *Head to tail*	[80]
Hylocereus polyrhizus	hylocerenin and phyllocactin	n-BuOH-ACN- water (5/1/6/ 0.7% TFA) TBMe-1-BuOH-ACN-water (2/2/1/5/0.7% or 1.0% TFA or (HFBA)	HSCCC model CCC 1000 (Pharma-Tech Research Corp., Baltimore, MD, USA) 850 mL; *Head to tail*	[73]
Phytolacca americana	betacyanins and betaxanthins	n-BuOH-ACN-water (5/1/6/0.7% TFA)	HSCCC model CCC 1000 (Pharma-Tech Research Corp., Baltimore, MD, USA) 850 mL; *Head to tail*	[79]
Celosia spicata (Thouars) Spreng	amaranthin, betanins, acylated amaranthin	TBMe-n-BuOH-ACN-water (2/2/1/5/0.7% HFBA); EtOH-ACN-n-PrOH-$(NH_4)_2SO_4$sat-water (0.5/0.5/0.5/1.2/1.0)	HSCCC AECS QuikPrep J-type hydrodynamic chromatograph (London, UK); *Head to tail/ Tail to head*	[82]

2.3 CHITIN INTRODUCTION

2.3.1 Chitin – a common nitrogenous polysaccharide

Chitin is a natural polymer found in 1884, and is known as the second most abundant polysaccharides on the earth next to cellulose. The compound appears plentiful in arthropod species such as shrimp (*Penaeus vannamei, Penaeus monodon*) and crabs (*Chionoecetes japonicas*)[86] which accounts up to 15-40% of total weight of these shells depending on origins, species and season. Chitin is also found available in the exoskeleton or cell walls of some specific insects, fungi or yeasts and several living organisms in the lower plants. The polymer displays crystalline inner structure and takes part in reinforcement and protection of the marine species. Nowadays, the chitin is produced commercially due to the steady supply of shrimp and crab shells from marine industry. The biodegradable properties, antibacterial activities and low toxicity for mammal's gastrointestins allow the polymer to be used as a food grade chemical.[87,88]

Structurally, these large molecules occur as straight co-polymers made of two constitutional units, mono *β*- (1→4)-2-acetamido-2-deoxy-*β*-D-glucopyranose and *β*-(1→4)-2-amino-2-deoxy-β-D-glucopyranose. This configuration is similar to that of cellulose and makes the compound significant water binding capacity of up to 673 - 805%.[88] The intra- and intermolecular functional groups namely hydroxyl (-OH), amine (-NH_2) and acetamino (-$NHCOCH_3$) cause the molecules very rigid and stable with water and ordinary organic solvents (except *N*,*N*-dimethylacetamide (DMAc)-LiCl, hexafluoroacetone or hexafluoro-2-propanol solution). These functional groups are also responsible for chitin physicochemistry, especially the percentage of *β*-(1→4)- 2-amino-2-deoxy-*β*-D-glucopyranose units present in the polymer chains, the so-called degree of acetylation (DA). If DA is less than 50%, chitin becomes chitosan - the most common derivative of chitin. Chitosan is soluble in light acidic solution and even in water if the DA is low enough; that high solubility made chitosan more important for some aqueous reactions.[89] There are studies showing the statistical differences in functional properties of chitin/chitosan originated from different species as well as from different production methods.[90] Figure 2.9 illustrates the structural correlation of chitin, chitosan and DA.

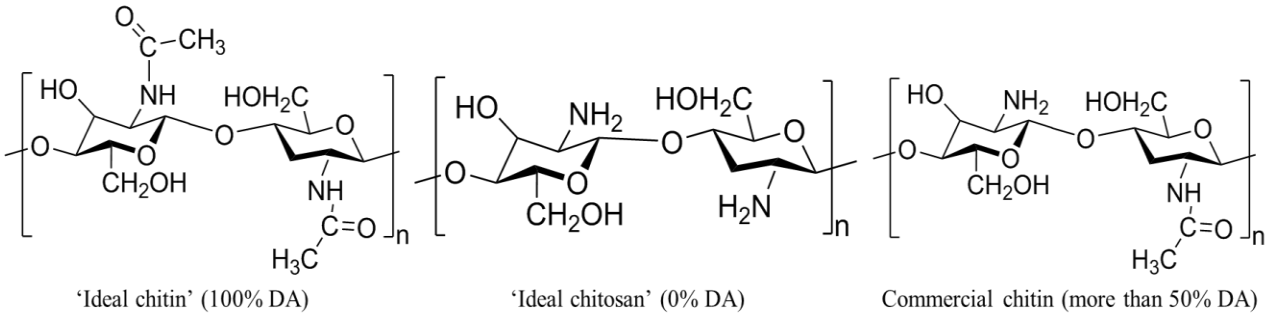


Figure 2-9: Chitin and chitosan structures correlated with degree of acetylation (DA)

2.3.2 Chitin production and application in industry

The yearly production of chitin is approximately 10^{10}–10^{12} tons based on the infinite raw materials source from the seafood industry. Chemical extraction is the most common method for producing chitin from Crustacean shells, consisting of several sequential steps from raw material preparation (washing/grinding/sieving), demineralization, and deproteinization to the final step to dry the product. The demineralization is done by aqueous HCl to liberate calcium carbonate and metal salts, whereas the deproteinization is conducted by aqueous alkaline solution (NaOH or KOH) at high temperature to break the strong covalent glycosidic bonds with proteins. That process results in chitin with 0.9-0.95% DA from the shrimp shells while more concentrated alkaline treatment causes further de-acetylation to form chitosan. Lately, the biological method is introduced to minimize the polluted water from the chemical process, in which the de-proteinization and demineralization are done by microbial fermentation (*Lactobacillus, Pseudomonas aeruginosa* K-187, *Bacillus subtilis*) or by natural proteases (Flavourzyme, *L. pentosus*).[91,92]

Chitin exhibits high insolubility, low chemical reactivity and non-toxicity (LD_{50} of chitosan in mice is comparable to sugar of 16 g/kg body weight), so they are well known as resins for water filtration. In industry, these polymers are used to eliminate heavy metals and industrial pollutants such as silver thiosulfate complexes, actinides, reactive textile dyes, etc.[93] These polysaccharides are also used in affinity chromatography as adsorbents for flavonoids, biflavonoids, amino acids, nucleic acids and phenolic glycoside separation.[94,95] Recently, chitin has been used in pharmaceuticals for wound healing, encapsulation, enzyme immobilizers, biosensors, etc.; they are also taken part in cosmetics and fiber materials. Their remarkable antimicrobial and antifungal activities have been reported in agriculture for anti-fungal agents.[96] In food industry, many applications of chitin have been introduced such as novel clarifier in fruit juice processing, components for antibacterial, gel-forming agents and protein accumulation in milk production. Interestingly, chitin and chitosan are proved in lab-scale having stabilizing activities to protect

polyphenol compounds against browning and over-oxidative effects, which negatively alter white wine. The capacity to reduce the mycotoxin ochratoxin A in red wine was also found in vitro based on their high affinity to these substances.[97,98] Additionally, there are some studies showing the strong binding capacity of chitin/chitosan on the natural pigment anthocyanins from reddish or *Perilla frutescens* leaves.[99,100] Recently, the strong interaction of chitin/chitosan to anthocyanin was intensively investigated, suggesting it as potential antioxidant carrier for colon treatment.[101] Noticeably, the application of chitin and chitosan on stabilization and extension the lifetime of the labile betalains by encapsulation has been reported.[102] Navarro et al. (2017) introduced the antimicrobial thin film made from chitosan-starch mixture modified by red beet extract; which could restrict the microorganism on both human and environment. This study could open the new chapter for food packaging and preservation to enhance the products shelf-life.[103]

2.4 BETALAIN MATERIALS USED IN THIS STUDY

2.4.1 *Opuntia spp.*

2.4.1.1 Opuntia spp. origin and distribution

Opuntia spp. is the dominant specie in the family Cactaceae - one of the most intriguing families spreading over arid and semi-arid regions. They originated in North/Southern America and entered as neophytes to the whole Mediterranean, Australia, South and North Asia, Africa. *Opuntia spp*. is considered as the representative plant in Mexico where 80 species and 150 cultivars are consumed.[11,104] The enlarging of desertification due to global climatical changes make them the favorite crops because of their high adaptation to dry agricultural conditions. Their mucilage structures, functional cuticles, crassulacean acid metabolism together with the shallow root system are claimed responsible for drought adaptation, which helps them to survive under insufficient irrigation. Nowadays, *Opuntia spp*. are found in Italy, Israel, subtropical African and American zones, Asia (China, South Korea) and even in Canada and Argentina.[105,106]

Figure 2-10: Vietnamese *Opuntia* cladods (left) and fruits (right) (source: https://cuuhocsinhphuyencom.wordpress.com/)

In pre-hispanic times, native american population used the plants for fruits and ancient medicines against diabetic and inflammatory diseases. Because of the limitation in nutritional information and the difficulties in postharvest, the plants were not commonly consumed until 1980s, when their phytochemistry and biofunctions were notified scientifically.[107] The edible parts including fruits and cladodes (pads, joints) contain high level of bioactive compounds. These micro-nutrients are varying depending on genus, environmental conditions and genetic diversity.

2.4.1.2 Health treatment effects

In the last decade, numerous scientific studies had been done on the correlation between the high nutritional values and health benefits of the cacti species. Recently, Díaz et al. (2017) summarized the bioactive compounds found naturally in a variety of *Opuntia spp*., and their therapeutic activities against chronic diseases such as atherosclerosis, cardiovascular diseases and cancer. These compounds including phenolic derivatives and dietary fibers being present in high concentration react as typical antioxidants and strong radical scavengers to prevent the oxidative stress and improve redox status within the plants.[106] In human body, they contribute to neutralization of reactive oxygen species, regulation of lipid and glucose metabolism which are necessary for anti-inflammatory activities, reduction of cholesterol and prevention of diabetes, obesity and cardiovascular diseases as well. In cancer therapy, cactus has been reported having cytotoxic effects on some cancerous cell lines, which could guide the direction for new anticancer strategies. In addition, the high content of minerals, amino acids, vitamins and betalain pigments strongly support the potential medication of these plants.[104,106,108]

2.4.1.3 Opuntia fruits: productions, appearance and composition

Over 30 countries around the world produce approximately 973 thousand tons of fruits per year, in which more than 50% come from Latin Americas where the fruit productions are highly seasonal and geographical depended. Nowadays, the fruits are popularly eaten fresh in the Mediterranean region, while the cladodes are eaten cooked as they supply dietary fibers useful to treat hypolipidemic.[109] Nevertheless, the soft texture and high sugar content make these fruits unstable representing by short shelf life of 3–4 weeks. That causes problems for these fruits under long-term storage and global distribution, and makes them only available constantly in local markets.

These *Opuntia* fruits (or prickly pears) appear in ovoid-spherical shapes with thick peels and many sharp prickles; the juicy pulp exhibits either deep red-purple, yellow or white color and distributing homogenously with the hard seeds. The fruit average weight is about 48-251g, in which peels, pulps and seeds are accounted for 28-58%, 37-67%, and 2-10%, respectively. The

pulps could contain up to 90% of water and 10-15% of reducing sugars (mainly glucose and fructose) whereas the soluble and insoluble fibers found more in peels and seeds. Recently, the pulp is not only consumed freshly but they are also used as natural pigment sources in other food products such as juices, jellies, jams, candies, ice cream and so on, due to the wide color hue caused by intensive betalains level.[110] Nevertheless, the incredible viscosity due to significant sugar and pectin contents hampers the fruit processing, and increase the loss of nutrition in waste materials. The raw material shortages and difficulties in processing make *Opuntia* fruits products not often available for food industry, therefore, more proper treatments are still required to enhance their application in food products.

Betacyanins and betaxanthins together with their stereoisomers are the principle pigments of *Opuntia spp*., in which betanins found in superior ratios than betaxanthins. The aglycone betanidin, acylated betanin namely phyllocactins, co-exist with their C15 isomers, are also found in lower quantities. These pigmented constituents in combination cause significant deep purple color, which displays a better color spectrum in comparison to that of red beet. *Opuntia spp.* betacyanins are more stable under different pH than that of red beets; that superior color stability makes this species the potential replacements for red beets in natural color production.[18,111] Figure 2-11 illustrates the structures of some main betalains present in *Opuntia spp*.

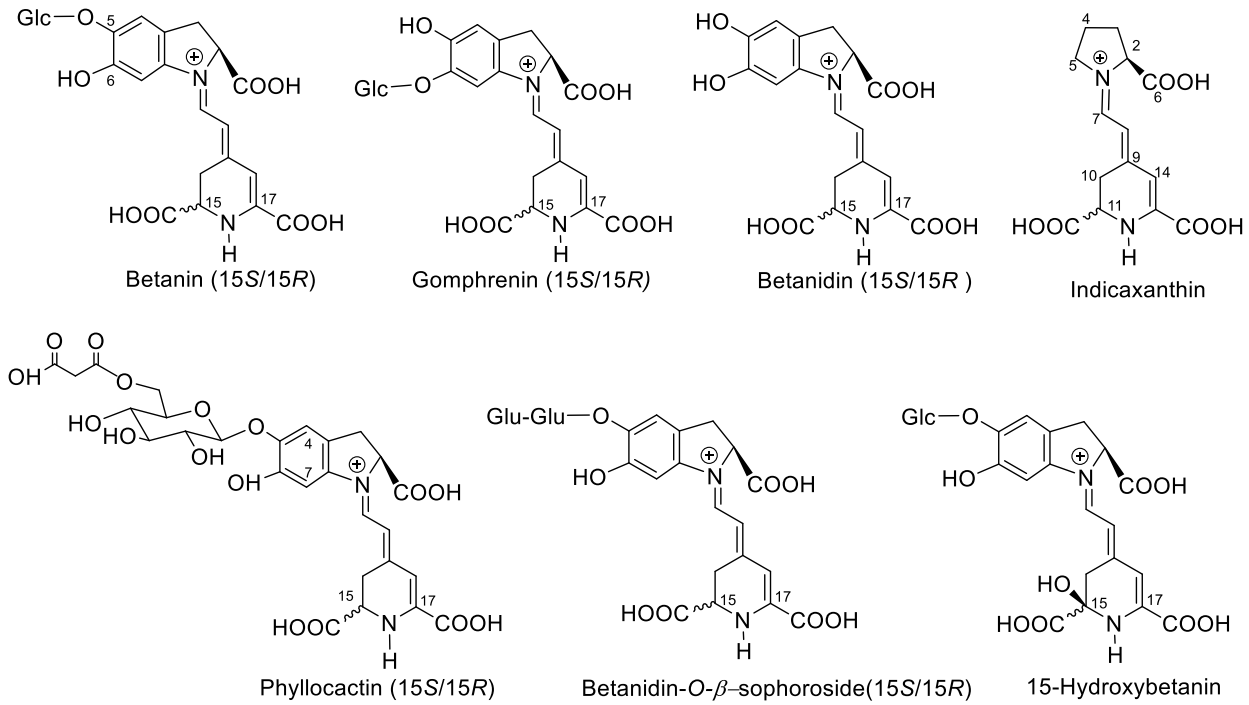


Figure 2-11: Structures of some main betalains occurring in *Opuntia spp*.

Additionally to betalain pigments, the significant amount of flavonoid glycosides and phenolic compounds were also recognized in these species; and the contribution of these functional

components to the bioactivities of fruits extracts had been proved. Quercetin glycosides and isorhamnetin glycosides are the two representative flavonoids which occur in the fruits at varied ratios depending on cultivars; e.g. isorhamnetin derivatives are dominant in *Opuntia-ficus indica* (*O. ficus*) while quercetins are abundant in *Opuntia lindheimeri*, *Opuntia strepthacantha* and *Opuntia stricta var. stricta.*[112,113]

2.4.1.4 Opuntia stricta var dillenii

Although *O. ficus* is the most common species among the highly consumed cultivars, *Opuntia stricta var dillienii* (*O. dillenii*) has been found as an alternative in food industry in terms of technical processing.[114] These two natural hybrid species are cultivated in Egypt but produce fruits in different seasons from June to September and November to January, respectively.[115,116] *O. dillenii* fruits appear in small shapes (10-20 g/fruit and 15-30 mm sizes) with highly mucilaginous deep red-purple pulp and small hard seeds covering by thick peels and thin skins. The juice has a plain acidic taste with pH around 3.6 and total soluble solids of approximately 10 Brix. Many reports have been published on the phytochemistry of this fruits showing superior antioxidant activities compared to *O. ficus*. For instances, *O. dillenii* contains more pigments and phenolics compounds, in which the betacyanin content is approximately 4 mg/g dry matter depending on extraction methods.[116,117] These pigments also exhibit a better resistance to environmental factors and processing conditions compared to red beet. The high ascorbic acid content results in lower pH fruits, and the constituents might work as the internal stabilizers for the pigments.[114] Jiménez-Aguilar et al. (2014) reviewed several common prickly pear cultivars in terms of betalain, flavonoids and bioactivities by different bioassays. They revealed that the genus *O. stricta* showed highest antioxidant activities compared to the others. On the other hand, *O. dillenii* extract are announced to be more active than that of *O. stricta* in others bioassays.[104,118,119,117]

2.4.2 Red dragon fruits (*Hylocereus polyrhizus*)

2.4.2.1 Origin, distribution and appearance of Hylocereus spp.

Dragon fruits or pitahaya are the popular names of several cactus species belonged to the genus *Hylocereus* under the Cactaceae family (Caryophyllales order). They are known as drought resistant species spreading globally. These species could adapt well to tropical conditions of long-term rain seasons and hot climates. There are 16 species from this genus cultivated as garden plants, from which only few of them produce large edible fruits (150-600 grams each). These species are also hybrids and developed as commercial cultivars for fruit consumption nowadays.[54,120] Dragon fruits originated in South America, then expanded to other

tropical/subtropical areas before commercially cultivated in many countries such as Spain, Mexico, Japan, Israel, Taiwan, Malaysia and Vietnam to date.[121]

Depending on the cultivars, these fruits appear in different colors such as yellow peels with white pulps (*Hylocereus megalanthus*), red peels with white pulps (*Hylocereus undatus*) or red pulps with red peels (*Hylocereus polyrhizus*).[122] The red skin cultivars have become popular globally because of their attractive color, fresh taste and high nutritional values. In Vietnam the fruits are called "Thanh long" and have been cultivated mainly in central and the south for self-supplying and also export (Figure 2-12). Vietnam is one of the top dragon fruit exporters supplying about 200,000 tons to the global market out of more than 600,000 tons harvested (2014); China, Europe and United States are their main export markets.[123]

Figure 2-12: Red and white pulp dragon fruits cultivated in Vietnam

In Vietnam, the most popular *Hylocereus polyrhizus* is a local specie developed from hybridization and plant selection from the red species originated in Columbia and the Vietnamese *Hylocereus undatus.* The fruits are produced nearly the whole year although the principle growing season is from March to October. This red fleshed cultivar is smaller in sizes with thinner peels in comparison to the white cultivar; that makes it shorter in shelf-life span compared to the white one.

2.4.2.2 Red dragon fruits nutrition

Although the white fleshed specie (*Hylocereus undatus*) has been more cultivated and distributed around the world, there is a rise in consumption of the red ones (*Hylocereus polyrhizus)* because of the outstanding pigmentation. The edible mucilaginous pulp is homogenously distributed with plenty of small soft black seeds, this part accounted up to 80% of fruits weight. At maturity, 82–88% of the flesh is water while the soluble solids concentration is 7 - 11 g/L. Glucose, fructose and recently sucrose are revealed as the dominant sugars, while citric acid and L-lactic acid display as the main acids (these actual concentration varied depending on ripening stages and

cultivating conditions). The ratios of sugar:acid range from 11:1 to 35:1 which are not preferable in sensorial evaluation and processing in term of thermal retention of the products, and additives such as ascorbic acid are added during dragon fruits processing to optimize that sugar:acid ratio.[124] Additionally, proteins and amino acids are also present in the fruits (0.23 g/100 g fruits) next to others minor composition such as lipids (0.61 g), polysaccharides, minerals (calcium, iron), vitamins (riboflavin, niacin and ascorbic acid), polyphenols and betalains pigments. This composition in combination make the fruit's distinct sensoria and the healthy low calories commodity.[122] However, the numerous small seeds together with high pectic and sugar contents hamper the processing of the juice and/or pigments extraction. These technical problems cause low yield and insufficient purification that limits the application of this fruit in food industry.[44]

2.4.2.3 Betalains phytochemistry and stability

The deep red/purple color of the dragon fruit pulps originated by high concentration of betacyanins of approximately 0.32 - 0.47 mg/g flesh.[125] These pigments consist of both non-acylated and acylated structures namely betanin, phyllocactin and hylocerenin in various proportions depending on cultivation and processing conditions; e.g. betanin could account up to 76% while hylocerenin contribute by 11.7% of total betacyanin. Each compound has been found co-existing with its C15 stereoisomer in different ratios due to their stabilities. For instance, betanin ranges from 18–76% compared to 3–14% of its counterpart isobetanin.[54] In addition, others minor pigmented structures are also elucidated from this fruits such as betanidin 5-O-β-sophoroside, 2'-apiosyl-betanin, sinapoylated apiosyl-betanin and so on. More than one betaxanthins were detected next to indicaxanthin, i.e., γ-amino butyric acid.[44,126] Noticeably, the peel also contains significant amount of pigments that makes these inedible residual a potential low cost-source for food colorants.[122] Figure 2-13 introduces several betalains structures found significantly in red dragon fruits.

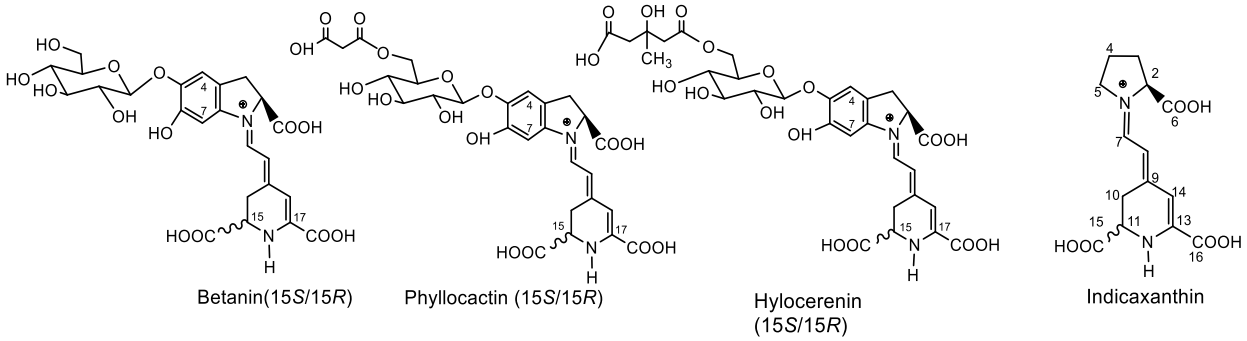


Figure 2-13: Betalains pigments of *Hylocereus polyrhizus*

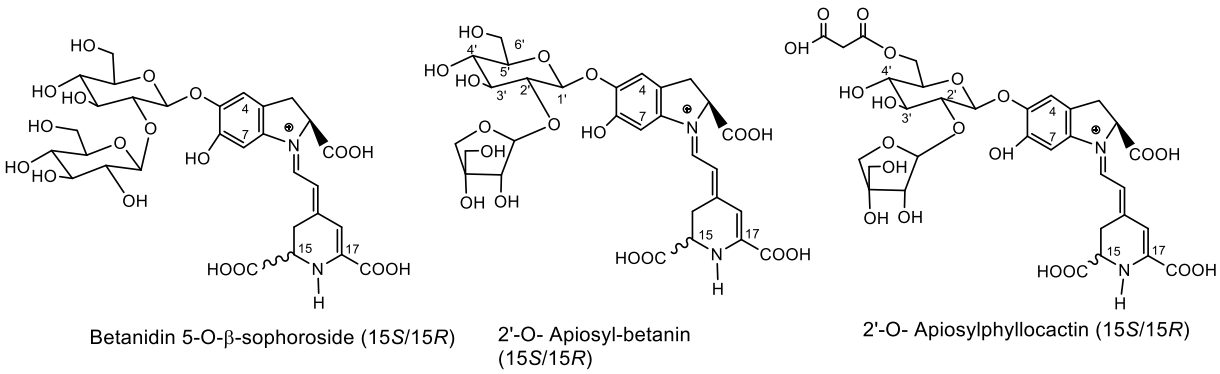


Figure 2-13 (cont): Betalains pigments of *Hylocereus polyrhizus*

Many studies have been done on the stability of dragon fruit pigments under various environmental and processing conditions. The stabilities of these pigments vary depending on their structural substitution according to Herbach et al. (2006), in which betanin is less thermal resistant than its acylated derivatives. The better heat retention of these structures is in co-relation to the high level of corresponding degraded structures induced during the treatments. Heating of phyllocactin release a mixture of degraded compounds exhibiting superior thermal stability compared to the initial phyllocactin.[127] The degradation pathway of the three principle betacyanins under thermal treatment were also suggested (Figure 2-14).[124,127] Beside the structure, the pH and composition of food matrices also influence the stabilities since they work as natural protection for these pigments. The existences of protective contributions were demonstrated by the higher vulnerability of the purified pigments compared to natural intact mixtures under processing conditions. Likewise, the addition of ascorbic acid or citric acid adjusting pH to lower values is known to prolong color stability of pigments in these products.[33]

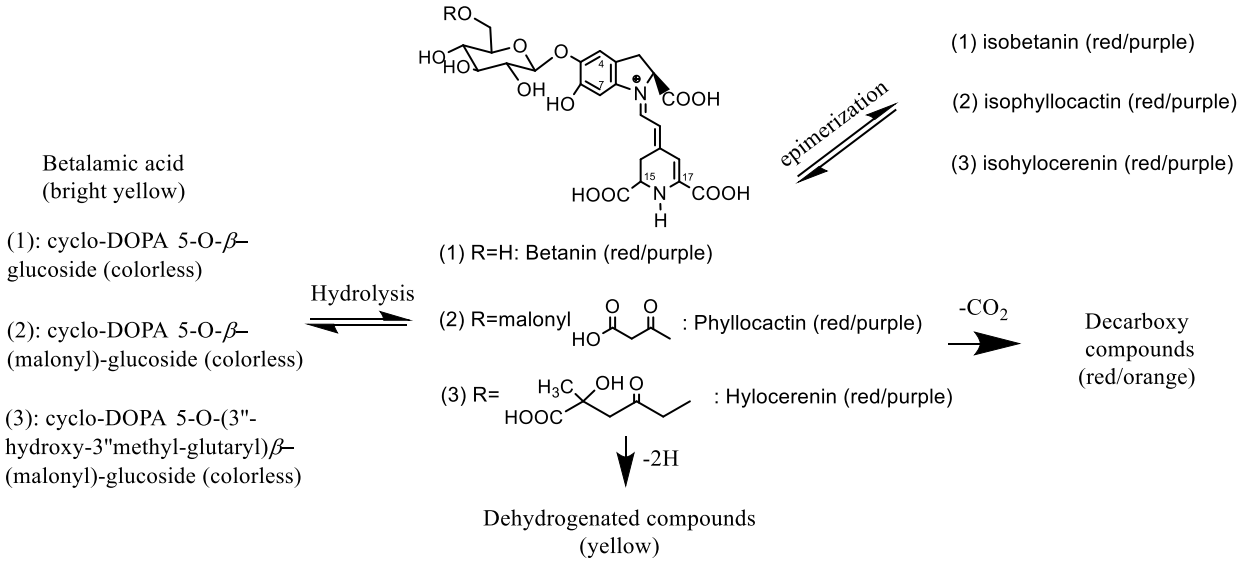


Figure 2-14: The thermal degradation pathway of dragon fruit betanin, phyllocactin and hylocerenin (modified from Herbach et al. (2006a))[127]

2.4.2.4 Application in food industry and potential health effects

The attractive colors of dragon fruits, their distinct textures and the high nutritional values are reasons to make them more popular and widely consumed as fresh fruits, more than application in food industry as colorant ingredient or the raw material for color extraction. Dragon fruits are present in many products such as ice-creams, jams, jellies, yogurts, milk products, light or soft drinks, etc. as a coloring source.[54] Recently, in Vietnam the dragon fruits are amongst the most important crops. There are new products commercially available recently such as wine and dried snack. The high betalain content suggests this cultivar as a potential replacement for red beets in the coloring market next to *Opuntia spp*.

The uses of dragon fruits as a healthy diet have been known in correlation to their low energy, high levels of vitamins and dietary fibers. These components are good for digestion and regulation of sugar level that contributes on reduction of cholesterol, blood pressure, diabetes and prevent colon cancer. Additionally, the significant levels of polyphenols and betalains are necessary for neutralizing the free radicals which could initiate human cancer.[120] In Vietnam, red dragon fruit is known as healthy fruit supplying only 40 kcal/100 g flesh with above 80% of liquid to prevent and cure constipation. Both peels and pulp exhibit strong bioactivities as antioxidant and radical-scavenging, which are comparable to that of red beets.[128]

2.4.3 *Atriplex hortensis var rubra*

2.4.3.1 Origin, distribution and appearance

Atriplex spp. belong to the Chenopodiaceae subfamily under Amaranthaceae family, which are known for their strong survival under both cold and hot climates. These genus do not require regular irrigation because they are highly resistant to drought; they also adapt well to salty and heavy metals contaminated soils. These distinguishable adaptations make them the largest and most diversified genus within this family. For instance, the deserts areas of North America, South Australia, South Central Asia, West and South East America, and the Mediterranean have been found as the principal regions of these species. Recently, global climate changes leading to the expansion of saline soils has suggested *Atriplex spp.* to be promising crops for both human and livestock due to their large biomass production. The plants have been cultivated as forage species on some dried areas where other crops are unable to grow. Noticeably, that great adaptation to harsh conditions makes this plant useful for the physiological and genetic study of stress resistance; some applications in cloning for drought and salt tolerated genes have been already introduced.[129]

Atriplex hortensis var rubra also known as red orach (sometimes red mountain spinach). The plant originated in Europe and Siberia, where they were cultivated as one of the oldest leafly

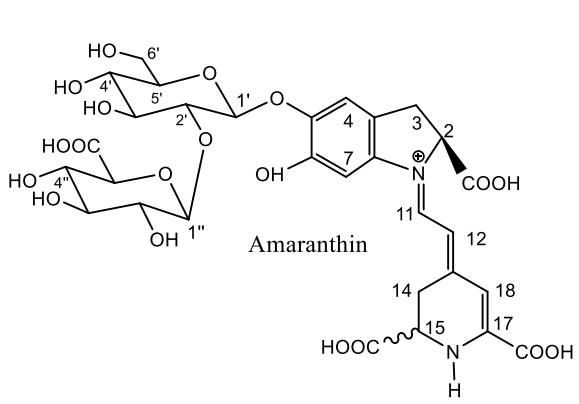
vegetables like spinach. They were also used as traditional medicines and soil erosion control specie. Nowadays, this salt bush widely distributes in many arid and semi-arid regions around the world. The plants occur with dark-red stems and leaves, no-petals flowers and small black seed covering by leafy membrane.[130]

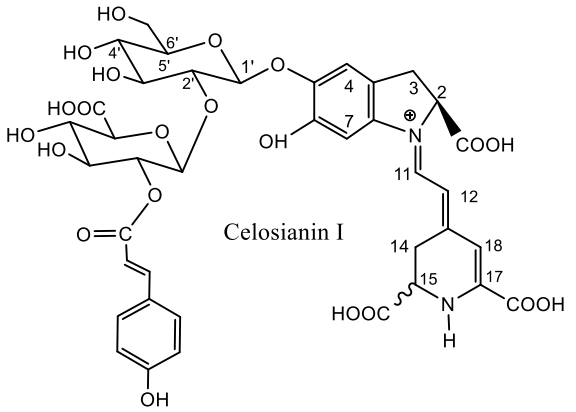
Figure 2-15: ***Atriplex hortensis var ruba*** **flowers and leaves** **(sources: httpwww.gardenersworld.complantsplant-finderatriplex-hortensis)**

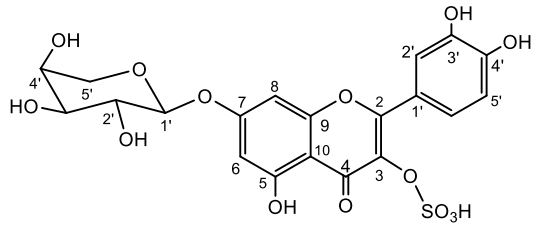
2.4.3.2 Phytochemistry and bioactivities

Only a few studies have been done on the compositions of this species, from which limited phytochemical information were reported. Vitamin A, saponins, oxalates, phyto-ecdysteroids, flavonoid glycosides, proteins, amino acids and minerals are found as the dominant bioactive constitutions; they contribute mainly to the metabolites of the genus, especially the high content of sodium chloride is responsible for the stress tolerance system of the plants. The concentrations of these chemical constitutes vary depending on diverse ecological regions.[131,132] These compounds have pronounced correlation with the positive effects of these species against human respiratory disorders, rheumatism, digestion, urinary diseases, nutritional absorption and enhancement of human body metabolism. These health benefits were known from ethno-medical ancient medications.[133]. Lately, research revealed the other contribution of these genus for gut treatments, antifungal, bronchitis, diabetes type II and anti-carcinogenic.[129]

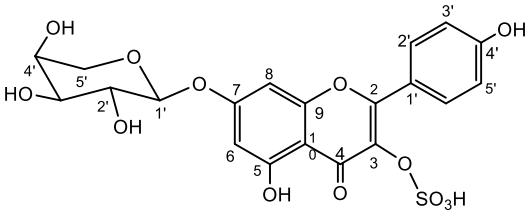
The high content of betalain such as celosianin II cause the deep purple color of the plants. The pigment class also displays a strong antioxidant capacity similarly to the distinct sulphated flavonoids elucidated from this species. These compounds have strong bioactivities such as oxygen species scavenging, metals chelating and enzymes interaction, adenosine receptor activities, etc. The uncommon sulphated flavonoids namely kaempferol-3-O-sulphate-7-O-arabinopyranoside, and quercetin-3-O-sulphate-7-O-arabinopyranoside together with their roles in biological regulation have been announced. Their biochemistry and treatment properties gained great interest as a new source of novel bioactive compounds.[133] Figure 2-16 presents structures of several pigments and flavonoids reported before from this cultivars.[85]







Quercetin 3-O-sulphate 7-O-α-arabinopyranoside



Kaempferol 3-O-sulphate 7-O-α–arabinopyranoside

Figure 2-16: Structures of several pigments and flavonoids existing in *Atriplex hortensis var rubra*

3. RESULTS AND DISCUSSION

3.1 CHITIN/CHITOSAN ADSORPTION CAPACITY TO BETALAINS: DEVELOPING A SIMPLE PRE-PURIFICATION METHOD FOR BETALAINS USING CHITIN

3.1.1 Aim of this work

The applications of chitin/chitosan on water treatment, pharmaceutical industry or agriculture have been known for years. Recently, the uses of these materials as chromatographic solid supports within natural compounds purification such as flavonoids, phenols, amino acids, inorganic ions and so on are noticed. However, the strong affinities between chitin and these bioactive compounds lead to irreversible adsorption and low yield recovery. This disadvantage makes these polymers not commonly used in separation technique.[134] Up to now, typical resins namely C18 reversed phase, Sephadex LH20, XAD-2, XAD-16, XAD-17 etc. are still used on the extraction or enrichment of pigments such as anthocyanins and betalains. Despite the high costs for these solid phase materials and a large amount of solvents required for desorption during recovery, these artificial resins are still preferred because of their strong interaction with the pigments. Nevertheless, these chemicals in combination with alcoholic solvents have been seen to enhance the degradation of betalains.[45,135] That raised a demand for an eco-friendly renewable adsorbent which is cheap, non-toxic, highly available and less harmful for the vulnerable pigments.

This work was done to look for a food grade resin having high adsorption capacity to betalains, from which a large-scale pre-purification process might be developed. Many ‘environmental friendly materials’ such as starch, egg shells, gelatin etc. were tested with commercial red beet juice, that resulted in only chitin/chitosan having significant affinities to the pigments. From then the comparison of betalains binding capacity to chitin and chitosan under both neutral and acidic pH was done, using commercial chitin, chitosan and *Hylocereus* pigments. However, chitosan tends to dissolve in acidic solution and is therefore not optimal for the pigments adsorption. Chitin treated with low pH (4-5), neutral chitin and neutral chitosan were used for betalains adsorption and desorption comparison experiments. All the samples were quantitatively analyzed by UV-Vis spectrophotometer, and data was processed by statistical differences test from Statistical Package for the Social Sciences-IBM Corporation software (SPSS). LC-ESI-MS/MS analysis was also used to compare the pigment profiles of adsorbed samples.

The enrichment procedure using chitin was then developed and applied to different betalain extracts namely red beet juice (fresh and commercial), *Opuntia* fruits, *Atriplex hortensis* leaves

and *Bougainvillea glabra* bracts. All adsorbed/desorbed samples were quantitative analyzed by UV-Vis spectrophotometer next to their original crude extract. That initial extracts together with desorbed samples were also submitted to LC-ESI-MS/MS to compare their pigmented constituents before and after the purification.

3.1.2 Binding capacity of betalains on chitin and chitosan under neutral and acidic pH

3.1.2.1 Comparison of the adsorption/desorption capacity of chitin and chitosan to betalains - quantitative evaluation by UV-Vis spectrophotometer

Figure 3-1 illustrates the adsorption (a) and desorption (b) of *Hylocereus* (dragon) pigment extract on neutral chitosan (left), neutral chitin (middle), and acidic treated chitin (right). As displayed, the white resins at the bottom changed from white to intensive red/purple color while the strong color of the supernatant faded after incubation. These color changes indicated for the strong pigments adsorption ability of the chitin/chitosan polymers. During the washing step, most of the unbound compounds were removed, the resins retained its violet color which correlated to their strong interaction to betalains.

Adsorption

Desorption

Figure 3-1: Comparison of dragon fruit pigments adsorption capacity on chitosan (left)/chitin (middle) and acidic chitin (right)

The *Hylocereous* pigment extract was used for quantitative experiments to determine the amounts of betacyanins bound to each sample (chitin neutral, acidic chitin and chitosan) measured from UV-Vis absorbance of the supernatants according to the method and equation (Eq. 3-1) suggested by Cai and Corke (1999):[136]

$$\text{Betacyanin concentration (mg/L)} = (A*F*MW*1000/\varepsilon.L) \quad \text{(Eq. 3-1)}$$

in which A is the absorption value at λ max (535 nm), F is the dilution factor, MW is the molecular weight of betanins (550 g/ mol), ε is the molar extinction coefficient of betanin (60,000/ mol.cm), and L is the path length of the cuvette (1 cm). Every sample was emplemented three times and measured in triplicate. The results are displayed in Table 3-1.

Table 3-1: Adsorption/ desorption values of betacyanins on chitin/chitosan and chitin treated by pH

	Chitin (mg/mL)	Chitosan (mg/mL)	Acidic chitin (mg/mL)
Adsorption	0.018 (±0.000522)	0.021 (±0.0007)	0.027 (±0.0004)
Desorption	0.0047 (±0.00053)	0.0017 (±0.0002)	0.0046(±0.00008)

Statistical differences test showed significant ($P<0.1$) differences in betacyanins adsorption capacity of chitin, chitin treated at low pH and chitosan. For instance, at neutral pH, chitosan had higher binding capacity to betacyanins than chitin (1.17 times), while chitin treated with acid displayed the highest power (1.5 times higher than neutral chitin and 1.3 time higher than that of chitosan). With the initial pigmentation concentration of the crude extract (0.030 mg/ml), 1g chitin, chitosan and acidic chitin adsorbed approximately 0.18 mg, 0.22 mg and 0.28 mg of betacyanins, respectively. These results were in agreement with the work of Changetal et al. (2000), in which chitin exhibited less binding power than chitosan to anthocyanins from the *Perilla* purple leaves extract. The better capacity of chitosan was explained by its higher amount of free $–NH_2$ groups, which are supposed to act as the adsorption centers of the polymers.[99] However, these studies were opposite to the results of Gao et al. (2014) on the application of different DA chitin to radish pigments, in which the binding capacity was positively correlated to the DA level.[100]

On the other hand, the pigments desorption process using 0.15M ammonium acetate/ethanol (Table 3-1) also showed statistical differences amongst three samples. The results showed small differences in the concentration of betacyanins from chitin and acidic chitin's supernatants, while that of chitosan was almost three times smaller. That could confirm the stronger affinity of pigments to the chitosan compared to chitin and low pH chitin, that strong binding made pigments hardly to be recovered from adsorbed chitosan. Similar results were found in the study of Wang et al. (2017) on the binding capacity of chitin microspheres to anthocyanin. They suggested that the strong adsorption capacity was caused by hydrogen bonds between free amino groups and hydroxyl groups within chitin/chitosan and anthocyanins structures. The electrostactic attraction which was induced under acidic condition was also claimed contributing to that binding mechanism.[101]

3.1.2.2 ESI-LC-MS/MS evaluation of the supernatants from adsorption

a) The crude extract of *Hylocereus polyrhizus*

LC-ESI-MS/MS was then used to compare the betalain composition of the supernatants from adsorption/desorption aqueous samples close to the crude pigment extract. The LC-MS chromatogram of crude dragon fruit extract is shown in Figure 3-2, from which several pigments were identified by the characteristic signals $[M+H]^+$ at *m/z* 551 and *m/z* 389 (Figure 3-2a). For instance, the two principle signals appearing $[M+H]^+$ at *m/z* 551 indicated for betanin and it C15 epimer. The signals of phyllocactin/isophyllocactin exhibiting at *m/z* 637 were well detected together with the signals of 15*S*/15*R* 2'-apiosyl-betanin (*m/z* 683). Although hylocerenin/isohylocerenin (*m/z* at 695) are known as the typical pigments of this cultivar, their appearances within this sample were less dominant.[137,138,139] Likewise, indicaxanthin epimers occurred as the sole abundant betaxanthin despite the fact that there were other minor betaxanthins from this species which were described previously.

Surprisingly, the degraded pigments namely mono-decarboxy betanin (*m/z* 507) and decarboxy neobetanin (*m/z* 505) were found significantly in this crude extract while neobetanin was rarely detected; these degraded betacyanins were recognized by principle fragment ion at *m/z* 345 or 343 (Figure 3-2b). These alterated products were also known from previous reports of cacti fruits, noticeably, the higher ratio of 15*R*/15*S* betanin isomers was opposite to the low level of 15*R* epimer naturally appearing. That abnormal ratio of the two isomers might be caused by the fast epimerization happening quickly during extraction, and could be induced by the used solvents. Additionally, there were a lot of non-pigmented compounds such as fruity acids, sugars and other metabolites, which were not in the focus of this study. Interestingly, these 'non-pigmented' components were rarely adsorbed by chitin/chitosan and the majority of them were then washed away by water during multiple rinsing steps.

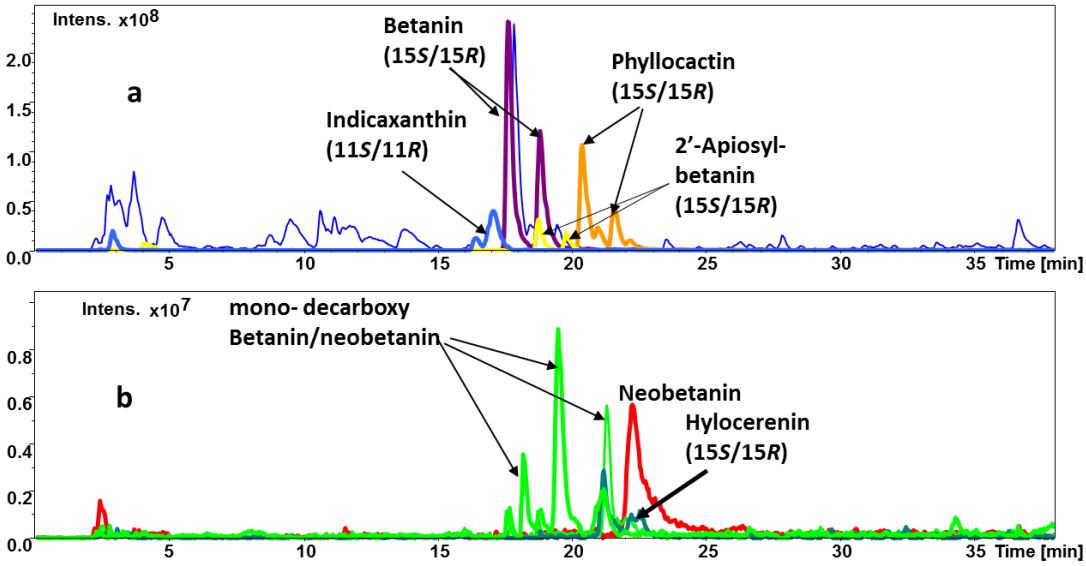


Figure 3-2: Modified LC-MS/MS chromatogram of dragon fruits crude extract (pos. mode)

b) The supernatants of chitin, chitosan and acidic chitin samples

The adsorption capacity of neutral chitin, neutral chitosan and acidic chitin were compared in term of principle pigments based on peak areas elucidated from ESI-LC-MS chromatograms of their supernatant described in Figure 3-1a. The results are displayed in Figure 3-3.

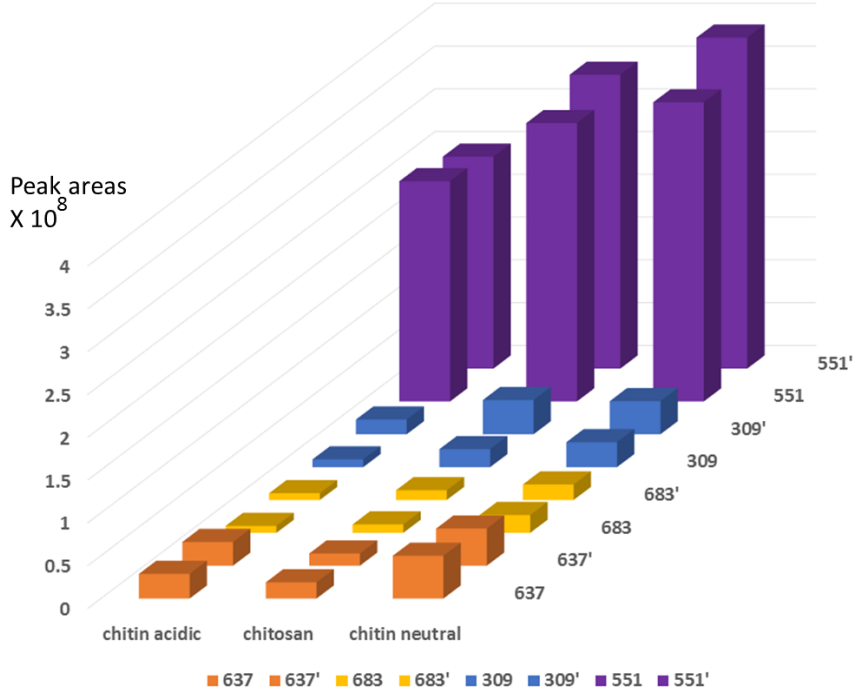


Figure 3-3: Betalains adsorbed on chitin/chitosan/acidic chitin elucidated from LC-MS

As it can be seen, the supernatants of acid treated chitin contained the lowest amounts of betacyanin pigments following by chitosan and chitin neutral. For instance, the low pH treated chitin material had trapped nearly 90% of betanin from the crude extract, compared to 86.5% of chitosan and 85.5% for neutral chitin. In case of 2'-apiosyl-betanin, the pigment concentration that remained in these supernatant were 11%, 13% and 27%, respectively. The results of indicaxanthin showed also highest binding ability for acidic chitin (90%) while that of chitosan was nearly equal to chitin (75% vs. 76%, respectively). As expected from the crude extract, the *15R* epimers appeared nearly equal to their 15*S* counterparts in most of the pigment solution; that were opposite to their natural occurrences in which 15*S* monomers are always dominant. The initial concentration of each pigments might affect the adsorption capacity of these polymers since the high concentration could oversaturate the adsorbed centers of the polymers.[93] For instance, the adsorption capacity of acidic chitin on betanin was lower compared to phyllocactin (90% vs. 97%) due to the overwhelming concentration of the betanin. This chitin pre-purified *Hylocereus polyrhizus* pigment mixture was then introduced to the CCC separation and will be discussed in detail in the following studies (section 3.5).

In general, chitin expressed lower affinity to betalains than chitosan while the treatment by acid strongly enhanced that adsorption capacity of chitin. On the other hand, the strong affinity of chitosan to these pigment resulted in low yield recovery of the adsorbed betacyanin compounds. Therefore, the acidic chitin was used to develop an optimized purification process for pigments from different betalain sources. That procedure was then applied on several fruits and vegetable to recover betalains in lab-scale which will be described in the next section.

3.1.3 The proposed adsorption mechanism of chitin/chitosan on betalains

Basically, commercial chitosan has less than 50% of DA compared to more than 90% of chitin; these differences in the number of free amino groups cause the distinct physicochemical properties of the two polymers. These functional groups of acetamino $–NHCOCH_3$ and amine $–NH_2$ in different ratio were suspected to be responsible for the adsorption mechanisms of the resins. This experiment compared the binding capacity of betalains to chitin and chitosan under neutral and acidic condition, since pH has been claimed to affect their physical behaviors such as swelling, solubility and adsorption capacity. For example, the enhancement of positive charges due to the stronger protonation of amine/acetamino groups under acidic environment were proposed accordingly to this reaction:

$$R\text{-}NH_2COCH_3 + H^+X^- \leftrightarrow R\text{-}NH^+_3COCH_3X^-$$

These positive charges could attract the components having negative charges or electronic abundant centers through electrostatic mechanisms, which were well described from the

affinities of chitin/chitosan to anionic dyes previously.[140] These results were in agreement to the study on chitin interaction with radish anthocyanins, in which their binding caused by electrostatic (hydrogen bonds and charge neutralization) and hydrophobic interactions.[101] Noticeably, when pH is above 4, the number of positive charges within anthocyanin structures are reduced; that enhances the electrostatic attraction to chitin instead of electrostatic repulsion. That might explain the strong interaction of neutral chitosan to betalains, resulting in the lower pigment recovery compared to neutral chitin and low pH chitin. In contrast, basic solution supplies with –OH groups that prevent these amines from protonation, and leads to low adsorption capacity of chitin to the pigments.[141]

The structures as phenolic compounds could embed strongly to these acetamino/ amine functional groups via hydrogen bonds due to their large number of hydroxyl group, resulting in significant adsorption capacity. Additionally, the hydrophobic interactions between chitin/chitosan amphiphilic structures with the hydrophobic compounds containing aromatic rings might also be an explanation.[142] For instance, the flavonoids C-glycoside from *Aleurites moluccana* significantly bound to chitin/chitosan via irreversible interaction of hydrogen bonds, electrostatic attractions and hydrophobic interaction.[94]

The adsorption mechanism of betalains to chitin/chitosan might explain similarly. The positive charge of betalains diazaheptamethinium partial structure could take part in that adsorption mechanism next to the van der Waals force.[101] Figure 3-4 introduced the proposed adsorption mechanism of chitin/chitosan on betalains.

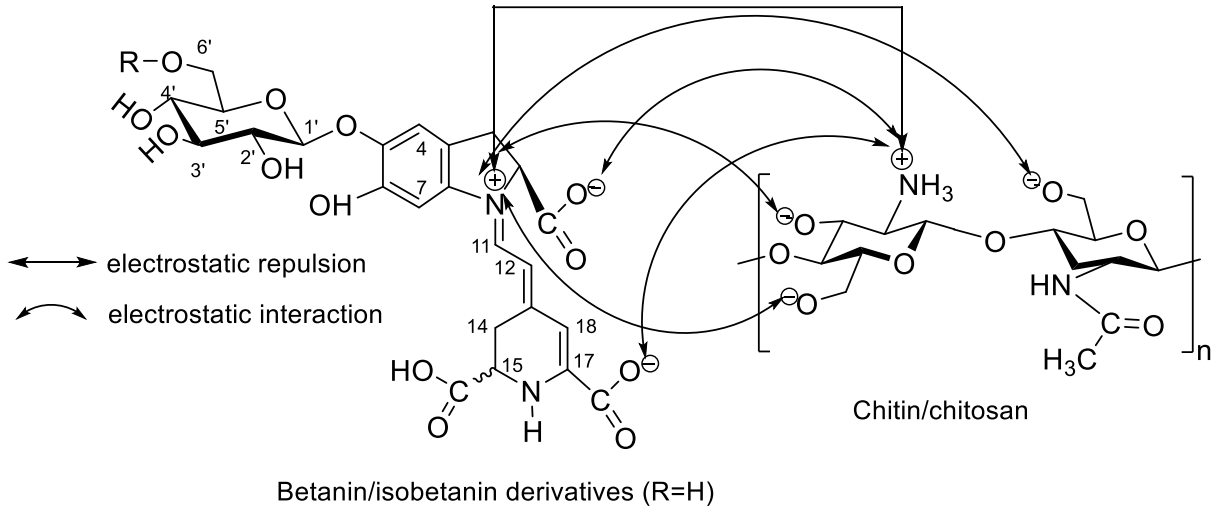


Figure 3-4: Proposed adsorption mechanism of betacyanins on chitin/chitosan

Under low pH (below 1.2), the electrostatic repulsion among the protonated amines was claimed responsible for desorption of anthocyanin since they enhanced the micropore size of the resin

structures. Likewise, the increase in the pigment desorption under high pH (above 7 to 9) was identical to the study of Chang et al. (2000), from which the limited amine protonation at high pH could weaken these electrostatic bonds.[100] However, betalains are unstable at pH above 7 that makes a high pH desorption not recommendable. The presences of strong ionic compounds were known to screen the electrostatic forces and led to the reduction of pigment binding capacity. Therefore, salt was added to the desorption procedure instead of alkaline.[101]

Besides, there were many studies on the effects of other parameters on the binding capacity of chitin/ chitosan next to pH and DA such as the initial concentrations of adsorbates, incubation time, temperature, the resin particle size, etc.[93,143,144,140] For instances, the adsorption of phenolic compounds on these biodegraded resins were reported depending on the surface area, porosity, functional groups, particle's size. This experiment indicated that chitin treated with acid could be potentially used for larger scale pre-purification of betalain pigments.

3.1.4 Chitin adsorption capability to betalains from a variety of betalainic materials

The complete purification process used in this part of study was introduced in Figure 3-5; this procedure was applied on fresh red beet juice, commercial red beet juice, *Opuntia* fruit juice and *Atriplex* leaves extract, respectively. All the crude extracts and the adsorbed, desorbed samples were quantitatively evaluated by UV-Vis and LC-ESI-MS/MS.

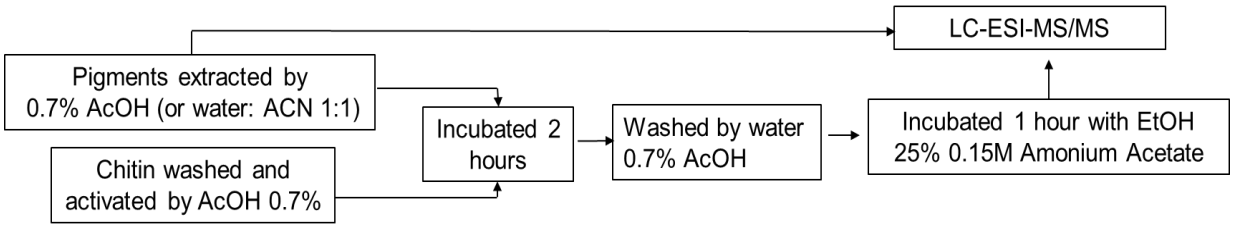


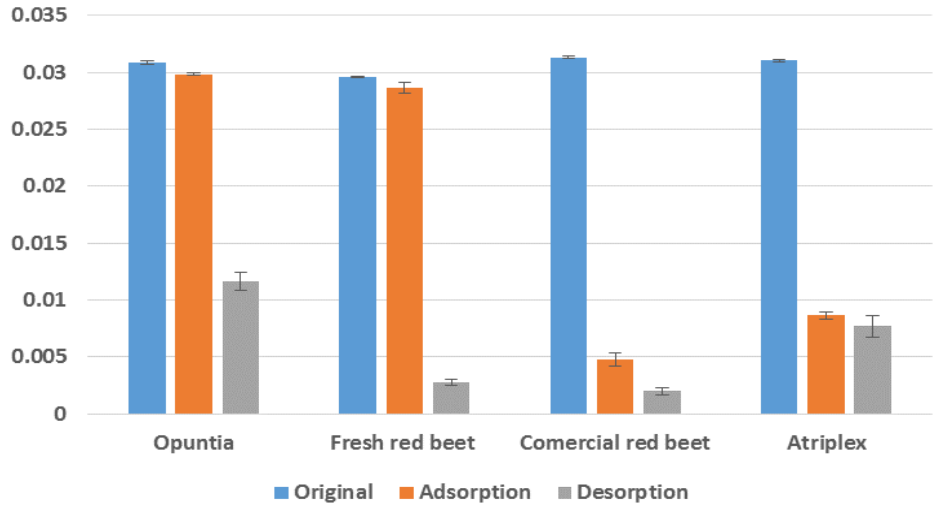
Figure 3-5: The adsorption/desorption procedure of betalains using chitin

3.1.4.1 Quantitative evaluation by UV-Vis spectrophotometer

The binding abilities of acidic chitin on several betalainic extracts were evaluated quantitatively and displayed in Figure 3-6.

As can be seen, the adsorption capacity of chitin was highest on *Opuntia* extract (approximately 0.30 mg/1g) followed by fresh red beet juice (0.29 mg/1g); the binding power of *Atriplex* pigments were 3 times lower than *Opuntia* but was much better than that of commercial red beet juice (0.08 mg/1g vs. 0.05 mg/1g) despite the fact that their original solutions had approximately equal concentration. The desorption showed distinct results in which more than 80% of the adsorbed *Atriplex* pigments were released, next to almost 40% of commercial red beet and *Opuntia* compared to only 10% of fresh red beet. Interestingly, the binding ability of chitin to red

beet pigments was very different between the fresh and the processed samples, which indicated specific effects of the pigment structures related to adsorption capacities. These different values in both adsorption and desorption processes made it worthy for further investigation by LC-ESI-MS in order to compare the components of each sample. That also indicated the requirement for modified adsorption/desorption processes accordingly to each betalainic source in order to optimize the recovery yields



Commercial red beet juice adsorbed by chitin

Figure 3-6: Binding capacity of chitin on betalain pigments evaluated by UV-Vis spectrophotometer and the fractions collected from purified commercial red beet

3.1.4.2 Pigment evaluation by LC-ESI-MS/MS-DAD

a) *Opuntia stricta var dillenii*

- Crude extract

The LC-ESI-MS chromatogram of *Opuntia* juice is displayed in Figure 3-7a and the principle pigments are summarized in Table 3-2.

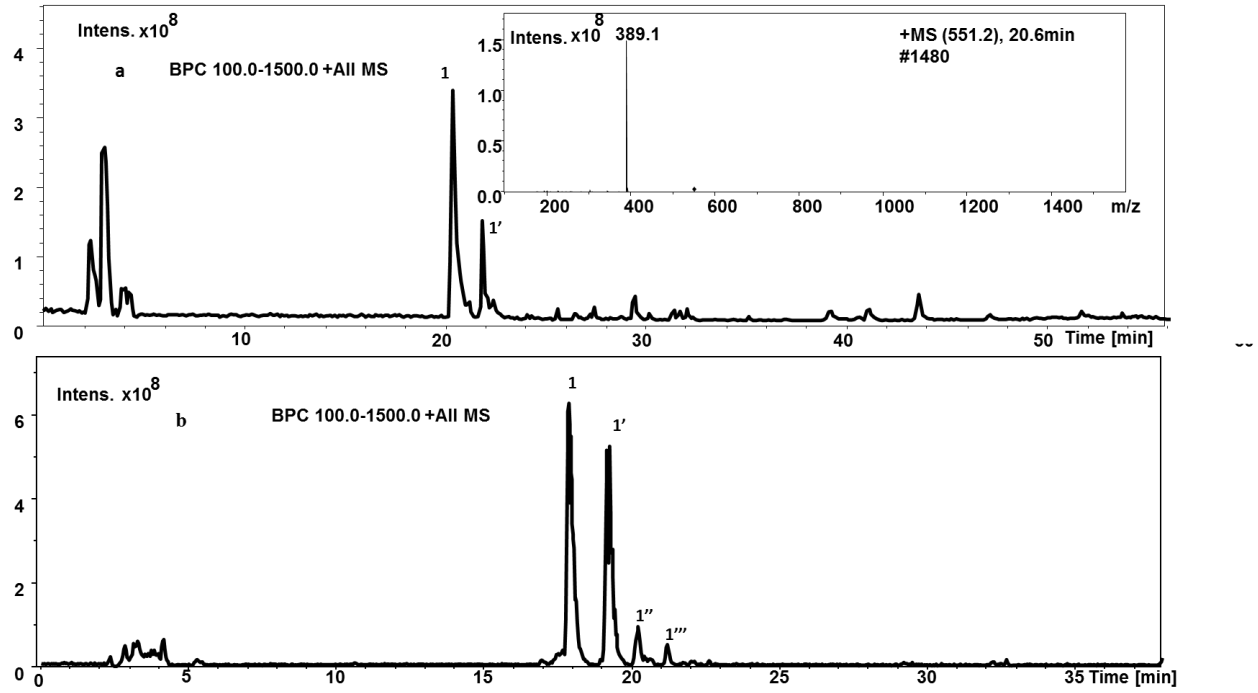


Figure 3-7: LC-MS chromatogram of *Opuntia stricta var dellini* crude extract (a) and enriched extract (b) (pos. ionization mode)

Betanin/isobetanin (**1/1'**) were the dominant betacyanins next to their 6-O-glycosidic positional isomers, gomphrenin I/isogomphrenin I (**1''/1'''**), displayed in lower intensites. The presences of these betanins epimers represented by the $[M+H]^+$ ion at *m/z* 551, and the cleavage of a glucose unit (Δ*m/z* 162) to the signal at *m/z* 389 were well identified in cacti fruits before.[145] Likewise, the signals of phyllocactin (15*S*/15*R*, $[M+H]^+$ *m/z* 637) together with feruloyl betanin (15*S*/15*R*, $[M+H]^+$ *m/z* 727) were also well recognized at lower ion intensities compared to betanins. Additionally, the betacyanin aglycone, betanidin ($[M+H]^+$ *m/z* 389), was detected in significantly amount in this crude extract. These pigments are the typical betacyanins of cacti fruits, which were found to be identical to the pigment profiles of *O. dellini* published previously.[45,139] The elution order of these betacyanins in the C18 reversed phase liquid chromatography depends on their polarities, in which betanin eluted first, followed by phyllocactin. Betanidin eluted later due to a more lipophilic structure and the loss of a glucose unit. The feruloyl betanin showed high retention time caused by the lipophilic feruloyl moiety. Meanwhile, the small signal of neobetanin was also recognized; this red-derivative is known as the indicator pigment of thermal treatment. Surprisingly, the hylocerenin pigment ($[M+H]^+$ *m/z* 695), a typical pigment of cacti fruits, was not seen in this cultivar. The eucomic acid existing within *O. ficus indica* fruits was also detected within this extract but the piscidic acid was not.[146] Besides, indicaxanthin was found as a sole betaxanthin at lower ion intensity. It intensively occurs in many *Opuntia* species next to other minor concentrated relatives as described before.[139] Other non-pigmented

metabolites were recognized as flavonoid glycosides: isorhamnetin-rutinoside ($[M+H]^+$ *m/z* 625), kaempferol-rutinoside ($[M+H]^+$ *m/z* 595), and several non-identified structures eluted lately. The natural product content of this crude extract was not very complex; however, the abundant polysaccharide content made it difficult for extraction process and required intensive purification steps to increase the pigment concentrations.

Table 3-2: The principles compounds from *Opuntia stricta var dillenii*

Name	λ max(nm)	$[M+H]^+$	MS^2
15*S* betanin/ isobetanin (15*R*)	520	551	389 (100)
15*S* gomphrenin I/isogomphrenin I (15*R*)	520	551	389 (100)
15*S* phyllocactin/isophyllocactin (15*R*)	538	637	551 (29.26), 389(100)
15*S*/15*R* betanidin 5-O-*β*-sophoroside	538	713	551(9),389 (82)
15*S*/15*R* betanidin	538	389	145(100), 345 (27)
cyclo-dopa-5-O-*β*-glucoside	282	358	196(100), 150 (40)
15*S*/15*R* feruloyl-betanin (gomphrenin III)	538	727	551(2), 389(100)
indicaxanthin	480	309	265 (100)
isorhamnetin-rutinoside		625	317(100), 479(15)
kaempferol-rutinoside		595	287(100), 449(16)

- The pigments purified by chitin

The LC-MS chromatogram of this *Opuntia* pigment extract enriched by chitin adsorption is illustrated in Figure 3-7b. In comparison to the crude extract, most of the interfering structures such as polysaccharides, acids and sugars were removed by the chitin clean-up, whereas all the betacyanins including betanin/isobetanin (**1/1'**), gomphrenin/isogomphrenin (**1''/1'''**), phyllocactin/isophyllocactin and feruloyl-betanin (or gomphrenin III) were highly accumulated. Surprisingly, the 15*S*/15*R* glycosyl-betanin ($[M+H]^+$ at 713) was found in significant level after purification by chitin although the appearance was hardly detected in the crude extract. The epimers were tentatively identified as betanidin 5-O-*β*-sophoroside and its isomers occurred in *Opuntia ficus*, *Hylocereus* species and *Mammillaria* fruits before.[126,147] A strong signal for indicaxanthin was also detected. Noticeably, a remarkable increase in the ion intensities of isobetanin to betanin was noticed in comparison to LC-MS from crude juice. This epimeric ratio change could be relevant to the strong isomerization under acidic condition; that was identical to betalain degradation pathway reported before.[31] Additionally, the trace of degradation products such as neobetanin, mono decarboxy-betanin and neo-decarboxy-betanin ($[M+H]^+$ *m/z* 549, 507 and 505, respectively) were also recognized. These pigment alterations might be generated during processing since their occurrences were not seen from the crude extract; and they were

displayed by the cleavage of a glucose unit to the decarboxy-betanidin or neo-decarboxy betanidin at *m/z* 345 and 343, respectively.

This result confirmed the high pigment content of this *Opuntia* species accompanied with a very high polysaccharide load, that makes the extraction/enrichment problematic and very time consuming. The use of chitin on *Opuntia spp*. extract suggested a simple and fast procedure for betacyanin pre-purification being applied on a high sugar content matrices.

b) Fresh red beet juice

- Pigment profile of the crude juice

Up to now, red beet roots are still the main source for betalains due to its high pigment content (1% of weight based on dry matter). That makes the red beet belonging to the top nutritional vegetables with respect to their antioxidant capacity despite the discovery of other plants rich in betalains.[148] The pigment profile of the crude juice measured by ESI-LC-MS/MS is presented in Figure 3-8a and the main compounds are summarized in Table 3-3.

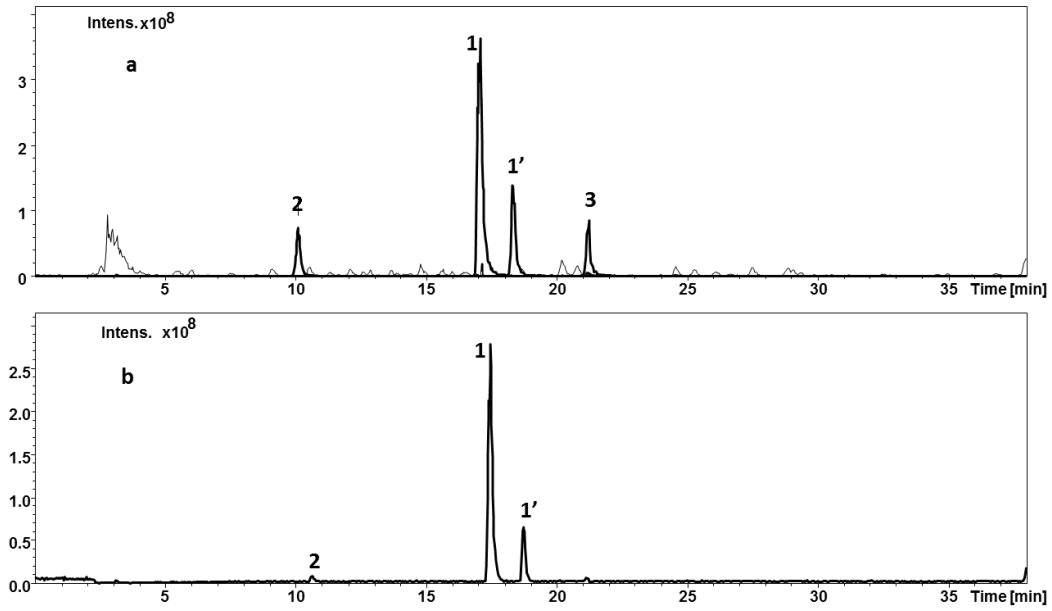


Figure 3-8: ESI-LC-MS chromatogram of the fresh red beet crude extract (a) and chitin purified extract (b) (pos. ionization mode)

As expectation from the previous experiments, the crude juice contained numerous water soluble compounds such as sugar and biopolymers, which were not of interest within this study. Betanin/isobetanin (**1/1'**) and vulgaxanthin I (**2**) were found as the principal pigments identical to the investigation of red beet.[22,36] In similarity, a trace of decarboxy-neobetanin ($[M+H]^+$ *m/z* 505) as well as decarboxy-dehydro-neobetanin (*m/z* 503) were found in the non-processed juice or other betalain-containing plants. In addition, the rare signals of betanidin (*m/z* at 389), cyclo-

dopa 5-O-*β*-glucoside (*m/z* 358) and other minor betalain derivatives such as sulphate prebetanin (*m/z* 631), 2'-O-glucosyl-betanin (*m/z* 713), 6'-O-feruloyl-betanin *(m/z* 727) together with their epimers were well detected. These pigments were recognized by their neutral loss differences Δ*m/z* in the MS/MS experiment to betanin (*m/z* 551) and betanidin (*m/z* 389), and were in good accordance to the previous work published on red beets.[22,40]

Table 3-3: Betalains profile of fresh red beet crude juice

Name	λmax (nm)	*m/z* $[M+H]^+$	MS^2 *m/z*
15*S* betanin/ isobetanin (15*R)*	520	551	389 (100)
neobetanin	520	549	387 (100)
prebetanin	538	631	551 (100), 389 (9.5)
vulgaxanthin I	483	340	323 (100), 277(22.6)
2'-O-glucosyl-betanin/2'-O-glucosyl-isobetanin	538	713	551 (8.87), 389 (81.7)
cyclo-dopa 5-O-*β*-glucoside	282	358	196 (100), 150 (40)
6'-O-feruloyl-betanin/6'-O-feruloyl-isobetanin	520	727	389 (100)

- Betalains profile of fresh red beet in chitin adsorbed pigment extract

Figure 3-8b illustrates the LC-ESI-MS chromatogram of fresh red beet juice enriched by chitin adsorption. While betanin/isobetanin were dominant pigments, low ion intensity of vulgaxanthin I were detected. The signals of prebetanin and 2'-O-glucosyl-betanin as well as 6'-O-feruloyl-betanin were only found in traces whereas most of the impurities were washed away. These results suggested that binding capacity of betalains on chitin not only depends on their initial concentration, but also on their structures which have specific affinities to the resin. This pre-purified process could be applied on larger scale, and the red beet enriched extract could then be fractionated easily by preparative-HPLC for pure betanins.

c) Commercial red beet

- Pigments of the crude juice

In the food and beverage industry, red beet is intensively processed by constant pasteurization or sterilization for storage and to extend the product shelf-lives up to six months. The significant changes in betacyanin pigment profiles under processing conditions and long-term storage were numerously studied before and showed similar results to this study.[40,149] For instance, betanin/isobetanin (**1/1'**) were still dominant pigments in which the 15*R* isomer appeared in higher level due to isomerization reaction enhancing under long-term thermal treatment. Drastic processing also generate typical heat-induced products such as various decarboxy dehydration betanins. The mono decarboxy betanins, namely 17-decarboxy-betanin/isobetanin (**2**/**2'**) and 15-decarboxy betanin/isobetanin were presented in this extract as expected.[40] Thermal treatment

induced the pigment dehydration and lead to neobetanin, which is considered as indicator for heating effects in betalainics fruits/vegetables.[150] Likewise, the decarboxy- neobetanin was also well detected as a side product of heat treatment next to signals of pigments precursor cyclo-dopa 5-O-*β*-glucoside (*m/z* 358) and betalamic acid (*m/z* 212). These highly polar compounds eluted early in the C18 reversed phase chromatography, which were identical to previous studies on commercial juice. Noticeably, the typical red beet betaxanthin, vulgaxanthin I, was not recognized from the profile. That could be related to the high instability of the betaxanthins compared to betacyanins under elevated temperature, which was well described previously.[22,138] Other unknown signals were detected in the beginning of the LC-ESI-MS chromatogram producing fragment ions correlating to sugars and oligomeric matrix components from the crude juice (which were not of interest in this study). All these compounds presented in a sum the dark red color of the thermally treated juice (Figure 3-5, right flash). The LC-MS chromatogram of commercial juice is presented in Figure 3-9a and the main betalains are summarized in Table 3-4.

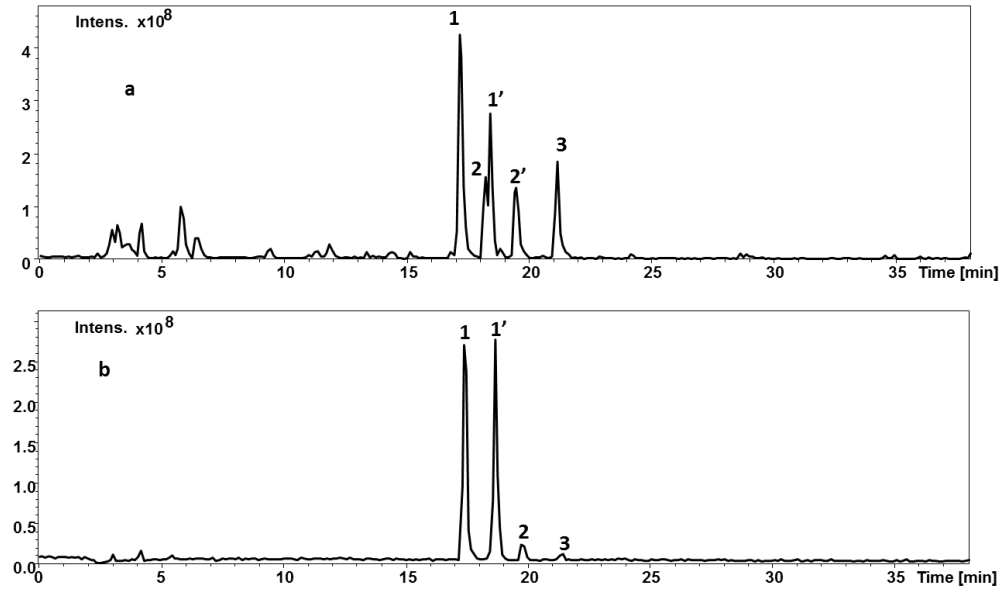


Figure 3-9: The LC-ESI-MS chromatogram of betalains in commercial red beet crude juice (a) and pigments purified by chitin adsorption (b) (pos. ionization mode)

- Pigments of commercial red beet juice purified by chitin (Figure 3-9b)

As proof of concept seen in LC-ESI-MS, betanin and its epimer (**1/1'**) were the main pigment compounds compared to the significantly low intensity signals of dehydro- (**3**) and decarboxy-derivatives (**2/2'**), most of the impurities of the crude extract were cleaned away. Likewise, the ratios of betanin and isobetanin were almost equal after the purification that indicated again an intensive betanin epimerization during processing which was found to be similar to the chitin

purification process of other betalain sources described above (*Opuntia* juice and fresh red beet juice).

Surprisingly, a majority of the decarboxy betanins was removed by the washing step; that suggested for the adsorption mechanism of these pigments on chitin. This material posses a very selective compounds recognition related to the amount of existing carboxyl groups in the pigment structures. The ionic interaction between carboxylic groups of betalains and the amine/aminic groups of chitin seem to be significant contributors to these adsorption mechanism. The mono decarboxy-betacyanins existing in high amount in commercial red beet juice were not effectively trapped by the bio-polymer chitin.

The red beet pigments are very important in food and pharmacy. Effective enrichment techniques on industrial scale are a prerequisite to remove high loads of sugar, nitrate, protein, salts, etc. from crude extracts. Many methods have been used to purify the pigments such as fermentation, liquid/liquid extraction and recently membrane separation technology, but none of them remove completely the impurities in a single step without hampering the pigment stability.[137] The application of chitin resulted in a high betacyanins concentration factor and reduced significantly the thermal degradation products of decarboxy-betacyanins and neobetanin within only one adsorption and desorption step using food grade chemicals as water, ethanol and ammonium acetate.

Table 3-4: LC-MS data of betalains in commercial red beet juice

Peak	**Name**	**λmax (nm)**	**$(M+H)^+$ *m/z***	**MS^2 *m/z***
1/1’	betanin/isobetanin	520	551	389 (100)
2/2’	17-decarboxy betanin/ isobetanin	535	507	345 (100)
3	neobetanin	520	549	387(85.2), 341(10.4)
-	decarboxy- neobetanin	538	505	343 (89), 297 (16.8)
-	cyclo-dopa 5-O-*β*-glucoside	282	358	196 (100), 150 (40)

d) *Atriplex hortensis var rubra* leaves

- Pigments from crude extract

The LC-ESI-MS chromatographic data of the crude pigment extract of *Atriplex hortensis* is illustrated in Figure 3-10a. A large number of compounds appeared at low elution times in the chromatogram, potentially related to non-pigmented compounds and were not furthered investigated in this study. The pigments were embedded in that complex mixture, in which two dominant betacyanins namely amaranthin (**1**, *m/z* at 727) and celosianin II (**4**, *m/z* 903) were well detected together with their C15 stereoisomers. Traces of minor pigments such as celosianin I (**3**,

m/z 873), betanin (**2**), the signals at *m/z* 889 (MS^2: 551, 727 and 389) and at *m/z* 859 (MS^2: 727, 683, 551 and 389), which were tentatively identified as betanidin-glucuronosyl-glucoside (amaranthin glucoside) and betanidin-glucuronosyl-xyloside (amaranthin xyloside), respectively, were detected.[74,80] Also, a degraded pigment such as decarboxy celosianin II (*m/z* 859, fragments at *m/z* 507 and 345) was recognized based on decarboxy aglycone betanidin (*m/z* 345). The flavonoid glycosides and sulphated flavonoids occurring naturally in this species were also found at the end of the chromatogram; these compounds were identical to the phytochemical study of this *Atriplex* genus described in literature.[129,133] These results showed the complexity of this crude extract, and the requirement of a pre-purification step to concentrate the pigments by eliminating most of the interfering structures.

- Pigments purified by chitin adsorption

The chromatogram of the extract purified by chitin is illustrated in Figure 3-10b and the principle pigments are summarized in Table 3-5.

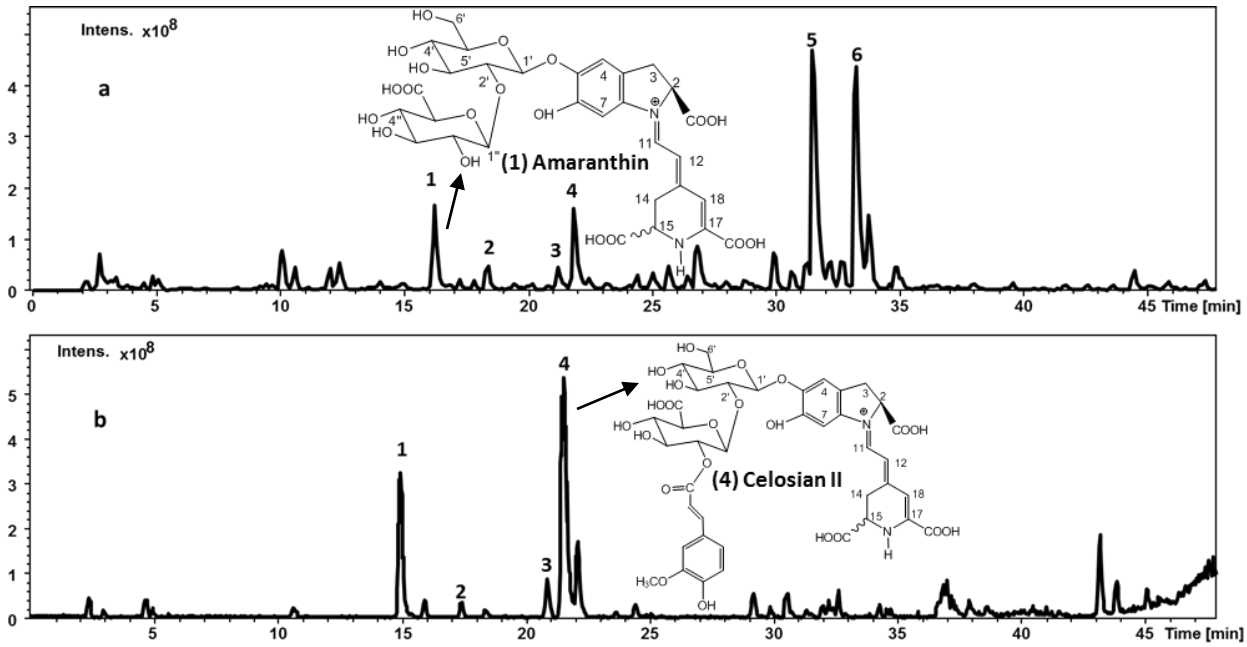


Figure 3-10: The LC-ESI-MS chromatogram of *Atriplex hortensis* crude extract (a) and chitin purified extract (b) (pos. ionization mode)

Table 3-5: Principle pigments from *Atriplex hortensis* leaves extract

Peak N°	Name	λmax (nm)	*m/z* $(M+H)^+$	MS^2 *m/z*
1	amaranthin	536	727	551 (7.3), 389(100)
2	betanin	535	551	389 (100)
3	celosianin I ((2"-O-E-4-coumaroyl)-amaranthin)	543	873	551 (9.6), 389 (100)

Peak Nº	Name	λmax (nm)	*m/z* $(M+H)^+$	MS^2 *m/z*
4	celosianin II ((2"-O-E-feruloyl)-amaranthin	530	903	551 (6.1), 389 (100)
-	flavonoid glycoside	-	551	302.7 (100)
5	flavonoid glycoside	362	535	287 (100)
6	sulphated flavonoid glycoside	362	499	367 (100), 287 (58.4)
-	flavonoid glycoside		419	287 (100)
-	sulphated flavonoid		439	421 (100), 394 (59.2)

In comparison to the crude extract, the LC-ESI-MS analysis of this purified extract showed that most of the non-betalain related compounds were washed away during chitin clean-up procedure, while the principal pigments were strongly concentrated similarly to the experiments above. For instance, the enhanced signals of celosianin II and amaranthin (15*S*/15*R*) next to that of betanin and celosianin I were in contrast to the significant reduction of signals **5**, **6** intensities. The neo-celosianin II, decarboxy celosianin II together with traces of decarboxy amaranthin were also detected as the results of the chitin purification step. Surprisingly, the traces of two minor pigments displayed at *m/z* 889 and 859 (amaranthin glucoside and amaranthin xyloside, respectively) were hardly recognized; the disappearances of these two compounds might correlate to the over saturation of the resin adsorbed centers due to the high concentration of the main pigment cellosianin II in the extract. Interestingly, the flavonoids occurred intensively in the crude extract were rarely found in this chitin eluted extract, which might confirm the adsorption selectivity of this resin to the betalain pigments. The *Atriplex* leaves clean-up procedure was then applied on larger scale to accumulate the pigments, particularly celosianin II for a preparative HPCCC separation, which will be discussed in details within the following section (Section 3.5).

e) *Bougainvillea glabra* bracts

- Crude extract

Since the betalains of these investigated fruits/vegetables showed this specific adsorption behavior to chitin, high molecular weight acyl-oligosaccharide betacyanins from the ornamental plant *Bougainvillea* bracts were also examined for their adsorption ability. The crude extract contained many pigments and non-pigment components in which the betacyanins eluted lately in the chromatogram because of their hydrophobicity compared to the derivatives in previous crude extracts. The varieties of acyl-oligosaccharide units and linkages generate the large number of high weight betacyanins.[80,151] The LC-MS chromatogram of the extract indicated the complex biochemistry; thus requiring an effective pre-clean/enriched process for further pigments investigation.

- Pigments purified by chitin

After chitin purification procedure, the *Bougainvillea* pigments were accumulated while the interfering matrices were almost removed. These un-polar pigments (*m/z* over 1000) eluted lately from the chromatogram which are displayed in Figure 3-11 with the selected ion traces of pigments of interest and summarized in Table 3-6. Surprisingly, the most concentrated pigment ($[M+H]^+$ at *m/z* 1005) was detected intensively in the chitin adsorbed extract although it was hardly detected in the crude extract. This pigments together with other high molecular weight compartments were found identical to the previous reports of Jerz et al. (2010).[80] That again confirmed the betalains adsorption capacity of chitin for highly complex betalain sources, and the specific binding behaviors among different betalain structures.

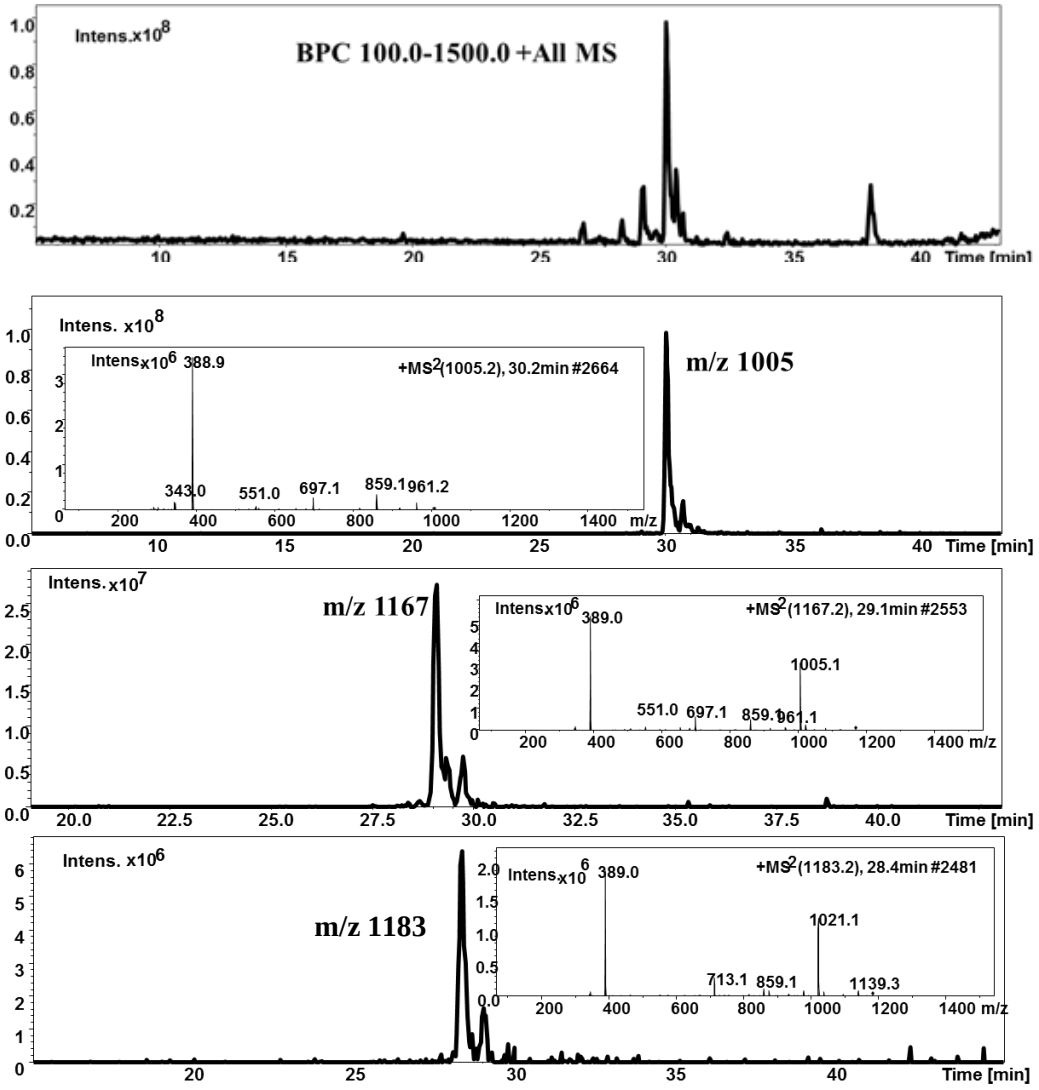
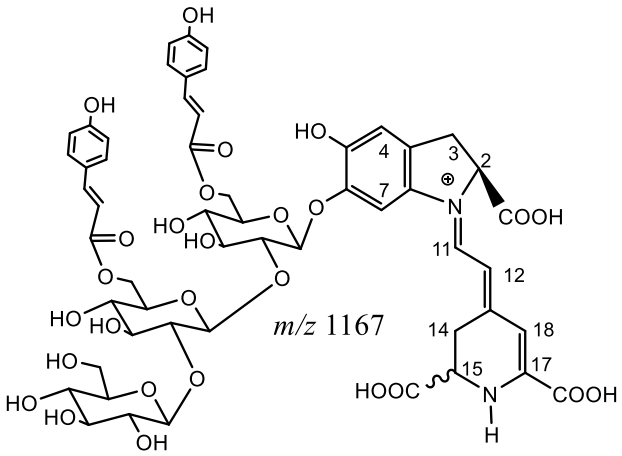


Figure 3-11: LC-ESI-MS chromatogram of the *Bougainvillea glabra* bract and selected ion traces of principal pigments purified by chitin (positive ionization mode)

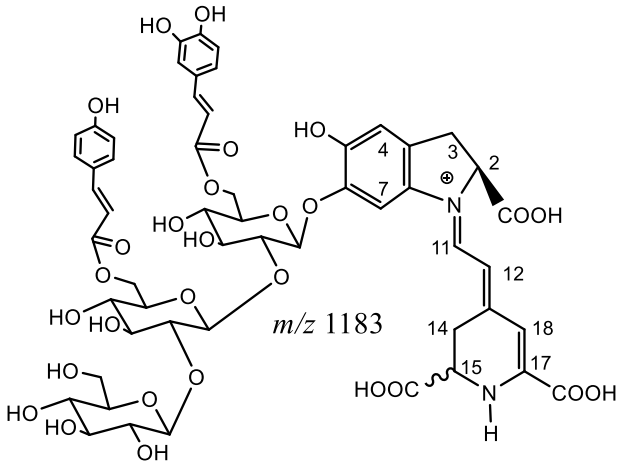
Table 3-6: Summary of the principal betacyanins existing in the examined *Bougainvillea glabra* bracts

Name	λmax (nm)	*m/z* $(M+H)^+$	MS^2 *m/z*
Betanidin-6-O-*β*-glucoside	520	551	389 (100)
Betanidin-6-O-*β*-sophoroside	537	713	551 (11.4), 389 (100)
Betanidin-6-O-(6′-O-*trans*-caffeoyl)-*β*-sophoroside	538	875	713 (18.6), 389 (100)
Betanidin-6-O-(6'-O-*trans*-4-coumaroyl)-*β*-sophoroside	538	859	713(5.0), 551(5.6), 389 (100)
(Glucosyl)-(coumaroyl-glucosyl)-betanidin	550	859	771, 713, 697, 551, 389 (100)
(Caffeoyl)-(feruloyl-glucosyl)-betanidin	513	889	713(2.4), 551(1.6), 389(81.6)
Betanidin-6-O-[(2''-O-β-glucosyl)-(6′,6″-di-O-*trans*-4-coumaroyl)]- *β*-sophoroside	558	1167	1005, 859, 697, 389 (100)
(Caffeoyl)-(glucosyl)-(coumaroyl-glucosyl)-betanidin	513	1021	977, 859, 713, 697, 398 (100)
Betanidin-6-O-{(2''-O-β-glucosyl)-[(6′-O-*trans*-caffeoyl)-6″-O-*trans*-4-coumaroyl)]}-*β*-sophoroside	513	1183	1021, 977, 875, 713, 389 (100)
(Coumaroyl-glucosyl)-(coumaroyl-glucosyl)-betanidin	550	1005	859(10.58), 697(5.94),389(100)
Betanidin-(Glucosyl) (feruloyl) glucosyl (glucosyl)	550	1035	859(10.58), 697(2.02),389(100)
Betanidin- Glucosyl (feruloy) glucosyl (feruloyl)	538	1065	1021, 889,727,551, 389(100)

Figure 3-12 introduces several structures of high weight molecular pigments detected in *Bougainvillea glabra* bracts.

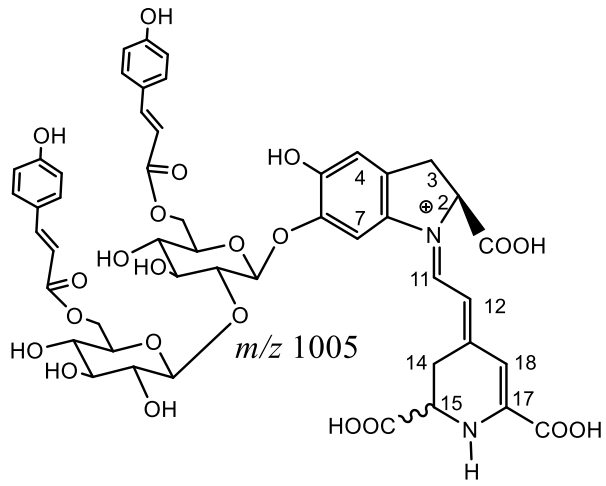


Betanidin-6-O-[(2"-O-*β*-glucosyl)-(6',6"-di-O-trans-4-coumaroyl)]-*β*-sophoroside

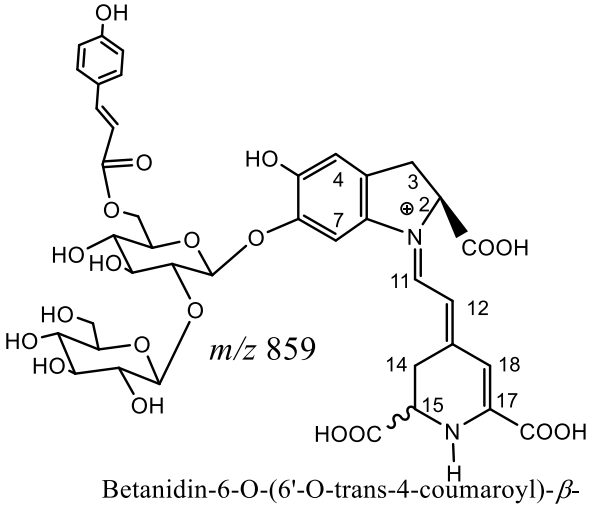


Betanidin-6-O-{(2"-O-*β*-glucosyl)-[(6'-O-trans-caffeoyl)-6"-O-trans-4-coumaroyl)]}-*β*-sophoroside

Figure 3-12: Betacyanin structures reported from *Bougainvillea glabra* bracts[80]



(Coumaroyl-glucosyl)-(coumaroyl-glucosyl)-betanidin



Betanidin-6-O-(6'-O-trans-4-coumaroyl)-β-sophoroside

Figure 3-12 (cont): Betacyanin structures reported from *Bougainvillea glabra* bracts[80]

3.1.5 Conclusions

Single factor statistical analysis showed that chitin, chitin treated by low pH and chitosan having distinct adsorption/desorption behavior on betalains, in which chitin at low pH appeared to have highest purification capacity. This method illustrated high binding capacity to the pigments and is suitable for the complex biomaterial matrices that could bring a new chapter for large-scale betalains production. The reversible adsorption allows the regeneration of these resin for both economic and ecological feasibilities, as they do not require large amount of organic solvents for high enrichment factor. Nevertheless, the adsorption capacity shows specific behaviors on different betalainic varieties; that requires more research efforts to optimize the adsorption/desorption parameters in order to get the highest pigment yields.

3.2 THE STUDY OF BETAXANTHIN SYNTHESIS FROM BETANINS PURIFIED BY CHITIN

3.2.1 Aim of this work

The bioactivities of betalain extracts were studied numerously whereas there were not many reports on that of single compounds, especially betaxanthins due to the insufficient pure pigments. Although betaxanthins exist in many betalainic varieties, the purification are not effective because of the highly instable nature of these yellow to orange color hue pigments compared to the purple betacyanin counterparts. Historically, the synthesis of betaxanthins by condensation of aldehyde group from betalamic acid with amine group from amino acids was discovered,[152] in which betalamic acid was produced instantly due to its high instability.[153]

Gandía-Herrero et al. (2006) suggested a protocol for betaxanthin synthesis including the betalamic acid generation through hydrolysis of purified betacyanins, followed by the condensation of generated betalamic acid with amino acids for the corresponding betaxanthin.[154] Lately, the novel one-step process was introduced from which the condensation happened on a solid bed of ionic polystyrene resin, where betalamic acid had been embedded.[155] Sekiguchi et al. (2010) reported that betalamic acid could be enzymatically produced from *Mirabilis jalapa L.*[156] and then could be condensed with amino acid for betaxanthins in vitro.

The aim of this work was to use chitin as a food grade and biological renewable resin for betalamic acid generation according to the work of Cabanes et al. (2014),[155] in which the pure betanin was adsorbed on chitin and hydrolyzed by alkali to release betalamic acid which then was reacted directly with amino acids for respective betaxanthins. The L-glutamic acid and L-proline amino acid were used to generate vulgaxanthin I and indicaxanthin, which were the two representative betaxanthins found in red beet and *Opuntia* species as described above. The hydrolyzed solution and generated betaxanthins were evaluated by LC-ESI-MS/MS. Figure 3-13 and 3-14 show the experiments and condensation reactions done in this study.

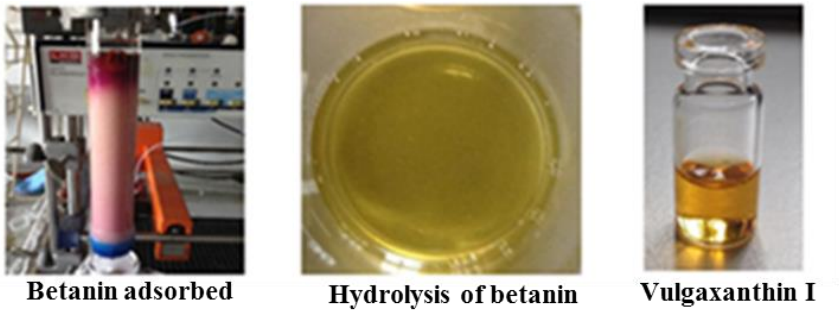


Figure 3-13: The main steps within betaxanthin synthesis procedure

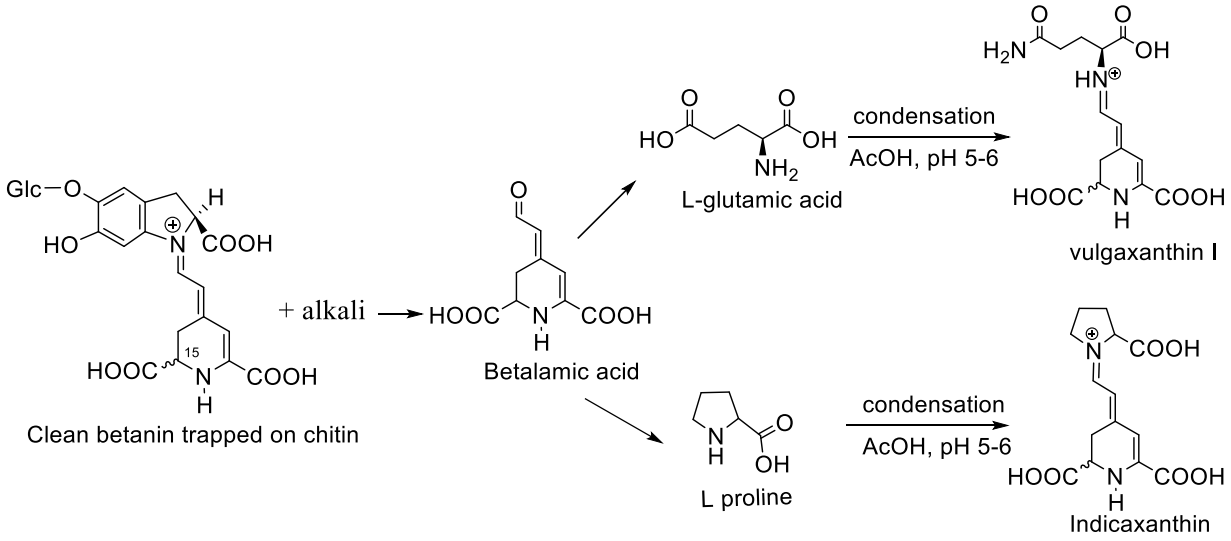


Figure 3-14: The condensation reaction of betalamic acid with L-glutamic acid and L-proline

3.2.2 The hydrolysis of betanin embedded on chitin resin

In this study, the commercial red beet juice was used as an initial solution of betanins as indicaxanthin and vulgaxanthin I were not present in this source (section 3.1.4.2.c). As already known from the previous experiments, the chitin procedure cleaned out the soluble contaminants such as sugar, amino acid and also most of the thermal mono/di-decarboxy degradation products whereas betanin/isobetanin retained on the resin together with a small residuals of neobetanin and decarboxy betanin (Figure 3-9). The hydrolysis was done by adding directly strong alkali solution (2M) to the adsorbed pigments; the elution released was evaluated by LC-ESI-MS (Figure 3-15) and the major degradation products were summarized in Table 3-7. A series of studies had been done on thermal degradation pathways of betalains; however, there were quite few reports on their alkaline degradation. During the strong alkaline treatment (e.g, ammonia hydroxide), betalains undergo hydrolyzation at the aldimine bond resulting in the break-down to precursors betalamic acid and cyclo-dopa-5-O-glycoside, and also a significant appearance of neobetanin due to dehydrogenation. That strong dehydrogenation was found similarly to the work of Skopinska et al. (2012) on the betanin photodegradation.[157] Besides, the small signal at *m/z* 547 (MS^2 at *m/z* 385 and 339) was also detected which identified as dehydro neobetanin. Additionally, the drastic alkaline environment caused cleavage of carboxyl groups releasing the decarboxy-betanin, decarboxy-neobetanin, decarboxy-2,3-dehydro-neobetanin which were observed in traces. These artifacts were identical to a report on the degradation pathway of betanins at low pH in which neobetanin, 2-decarboxy-2,3-dehydro-neobetanin and the C15 isomerization were clearly described.[158] However, more studies on the alkaline hydrolyzation of the pigments are needed to confirm these degradation products.

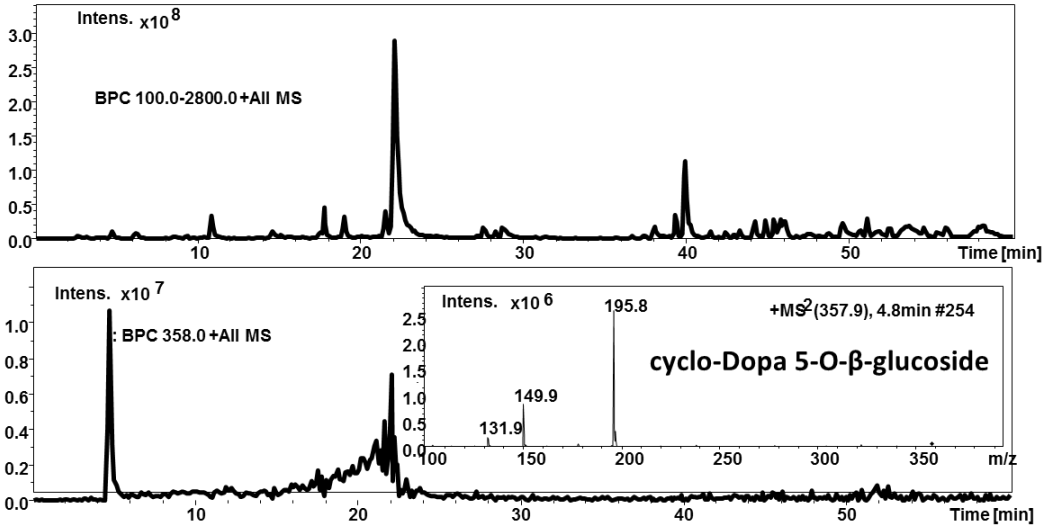


Figure 3-15: LC-MS chromatogram of the betanin hydrolysis eluting from a chitin column with selected ion traces of degraded compounds

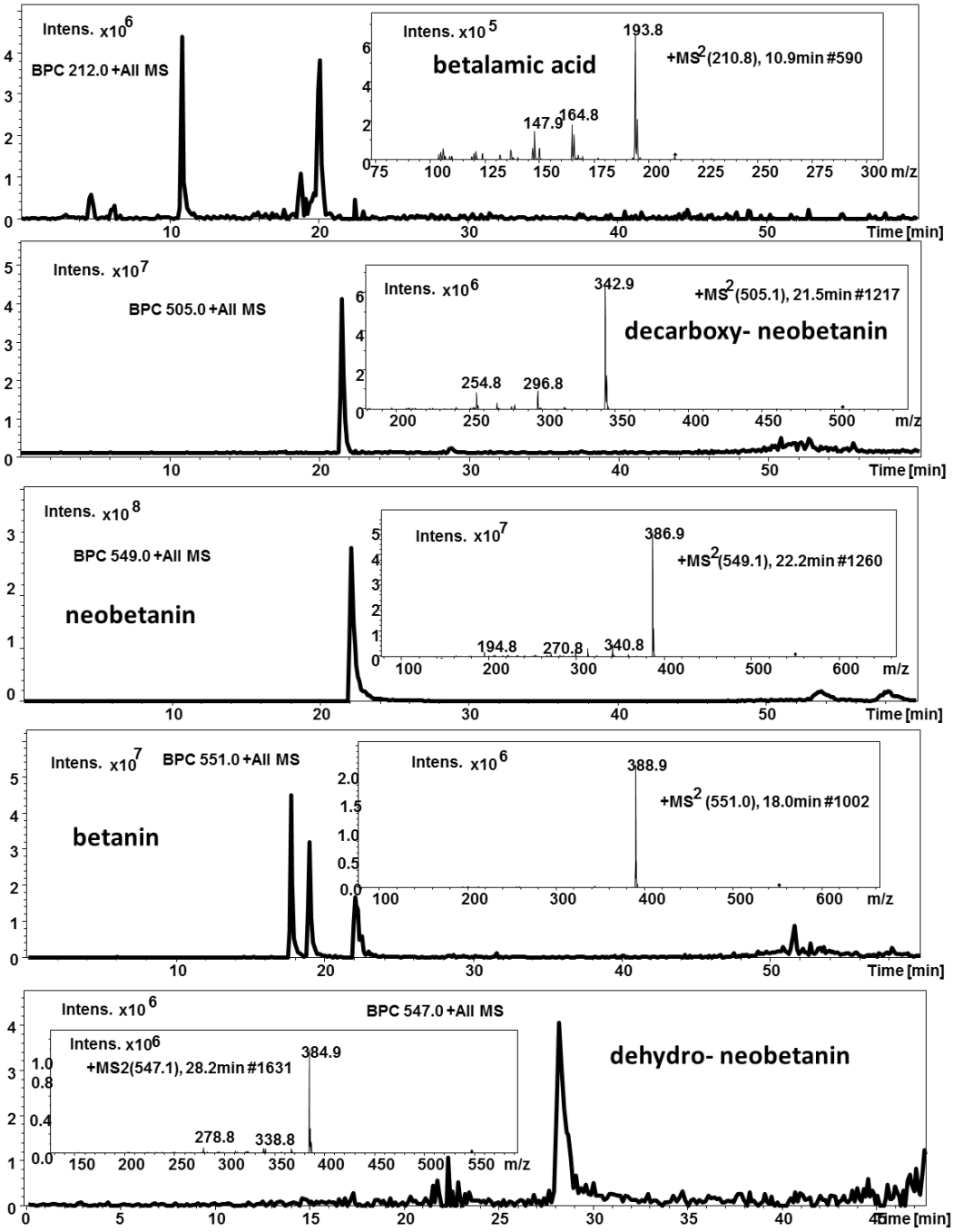


Figure 3-15 (cont.): LC-MS chromatogram of the betanin hydrolysis eluting from a chitin column with selected ion traces of degraded compounds

Interestingly, intensive signal of betanin/isobetanin were still detected next to low signal of betalamic acid suggesting for the regeneration of these pigments during processing. The slow recovery of betanin under low temperature (freeze drying, cold storages) was first observed by Huang and Elbe (1985). They proposed the mechanism of betanins regeneration through the Schiff base reaction of nucleophilic amine (cyclo-dopa) and aldehyde (betalamic acid) available

at high concentration under cold condition.[41] In addition, few signals of remaining impurities retained strongly on chitin were released due to the de-protonation of $–NH_2$ groups by strong basic environment. These components in combination with pigments caused a green/pale yellow color of the hydrolyzed eluate (Figure 3-13, middle). Noticeable, due to the differences in maximum absorbance wavelength and concentration of each components, the color of hydrolyzed mixtures and the resulting synthesized betaxanthin could be affected.

Table 3-7: The main hydrolyzed products of purified betanin pigment originated from commercial red beet juice

Name	λmax (nm)	*m/z* $(M+H)^+$	MS^2 *m/z*
neobetanin	470	549	387 (100), 341 (9.07)
betanin/isobetanin	520	551	389(100)
betalamic acid	437	212	194(100), 166(24.9)
cyclo-dopa 5-O-*β*-glucoside	283	358	196(100), 150(31)
decarboxy betanin	504	507	345(100)
decarboxy- neobetanin	476	505	343 (90), 297 (16,8)
decarboxy-dehydro-neobetanin	445	503	341 (100), 295
dehydro-neobetanin	488	547	385 (100), 339 (4.5)

3.2.3 Betalamic acid and amino acid condensation for betaxanthin synthesis

3.2.3.1 Vulgaxanthin I

Vulgaxanthin I and II are the dominant but instable betaxanthins occurring naturally in *Beta vulgaris*, especially the yellow pigmented cultivars. These yellow/orange pigments are more vulnerable to temperatures and low pH compared to betacyanins.[36] While most of the vulgaxanthins used in industry originate from the beet root species, there were protocols to synthesize these pigments for standard chemicals being used in analysis, despite the fact that these procedures were in low yields and also time consuming. In principle, vulgaxanthin I was produced from the Schiff condensation reaction of betalamic acid and glutamic acid.

In this experiment, the betalamic acid was generated from a fast and simple method using chitin as solid bed in which purified betanins were then hydrolyzed by strong alkali solution to liberate in-situ betalamic acid. The condensation was buffered by acetic acid to neutral pH to optimize the reaction and to prevent the regeneration of betanin/isobetanin and to enhance the resulting vulgaxanthins yield. During the condensation, the pale yellow color of betalamic acid solution changed to deep orange color (Figure 3-13, right) which was an indication for the generation of vulgaxanthin I. The presence of vulgaxanthin I was then confirmed by the LC-ESI-MS measurement with $[M+H]^+$ at *m/z* 340 and fragment ions at *m/z* 323 and 277 together with the absorption maximum at λ 480 nm; which were identical to the data of previous experiments.

Additionally, the decrease of signal intensity of betalamic acid also confirmed the reaction. Nevertheless, there were other compounds present such as betanin/isobetanin, neobetanin and decarboxy-neobetanin that reduced the reaction yield. Therefore, more quantitative evaluation is required to optimize the reaction for yield and purity improvement of betaxanthins partial synthesis. Figure 3-16 illustrates the LC-MS chromatogram of the synthesized vulgaxanthin I.

3.2.3.2 Indicaxanthin

This yellow/orange betaxanthin is known as the characteristic pigments of *Opuntia* species next to betacyanins; it is abundantly found within the yellow and white cultivars such as yellow *Opuntia ficus indica*. The pigment presence was found to contribute to the potential inhibition of gastrointestinal disorders and anti-inflammatory effects of the fruit extracts.[159] Indicaxanthin was proved to have the highest bioactivities amongst betaxanthins and these effects are comparable to that of quercetin.[156] In lab-scale, the pigment was isolated from *Opuntia* species through preparative HPLC or Sephadex column.[160] The pigment was also detected in yellow beet by LC-ESI-MS at the signal $[M+H]^+$ *m/z* 309 and fragmented to daughter ions at *m/z* 263 and 217.[146]

Basically, indicaxanthin was generated by the condensation of betalamic acid and the amino acid L- proline; that principle was done successfully within the synthesis of Trezzini and Zryd (1990) by using red beet betanins as betalamic acid supplement.[152] Later, 14 betaxanthins including indicaxanthin were released by a semi-synthetic procedure from betalamic acid using ionic solid purification; this procedure was able to produce the diastereoizomeric forms of indicaxanthins.[154]

Our experimental approach was to generate betalamic acid from chitin with adsorbed betanin for indicaxanthin. The LC-MS chromatogram of synthesized indicaxanthin is presented in Figure 3-17. Similar to the synthesis of vulgaxanthin I, neobetanin appeared dominantly next to the generated indicaxanthin. Interestingly, the signals of betanin/isobetanin were smaller than it was observed in the vulgaxanthin synthesis experiment indicating a limited regeneration. However, impurities appearing at early and lately elution time of LC-chromatogram showed that chitin meterial was not so pure and a better quality should be used for pigment synthesis. This experiment could be applied on a larger scale and further fractionation by preparative HPLC could lead to significant amount of highly pure indicaxanthin.

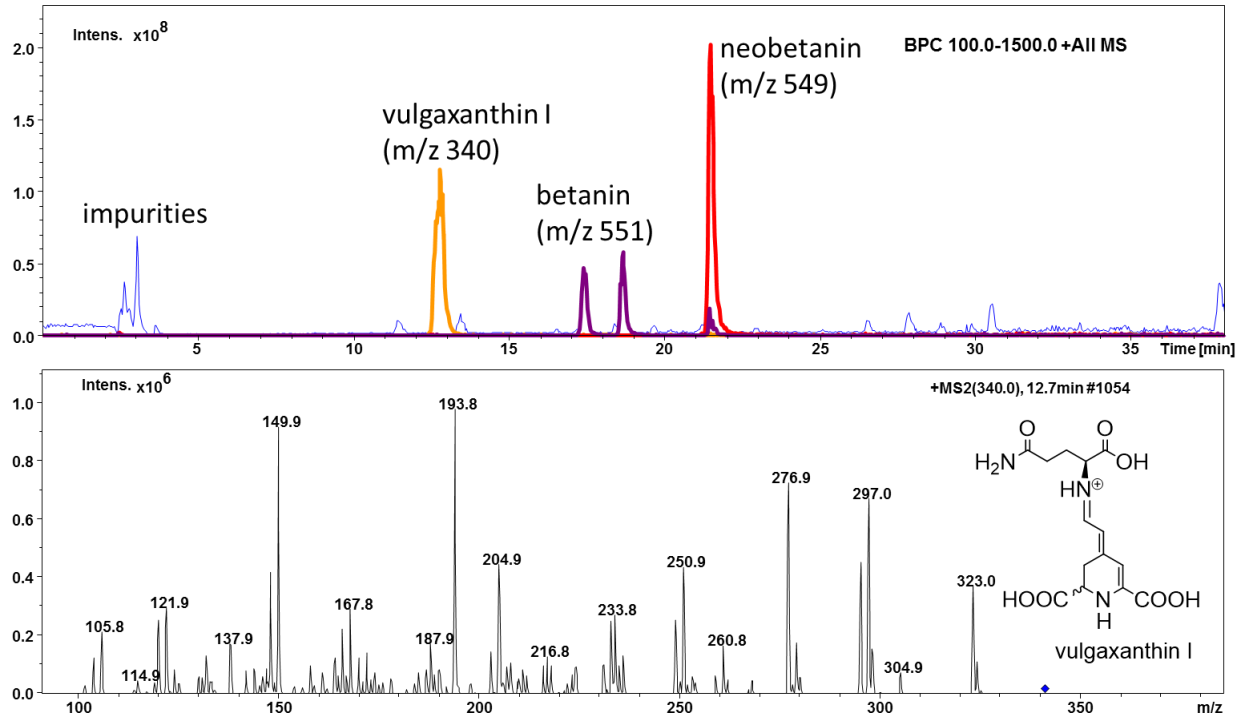


Figure 3-16: LC-MS chromatogram of the synthesized vulgaxanthin I

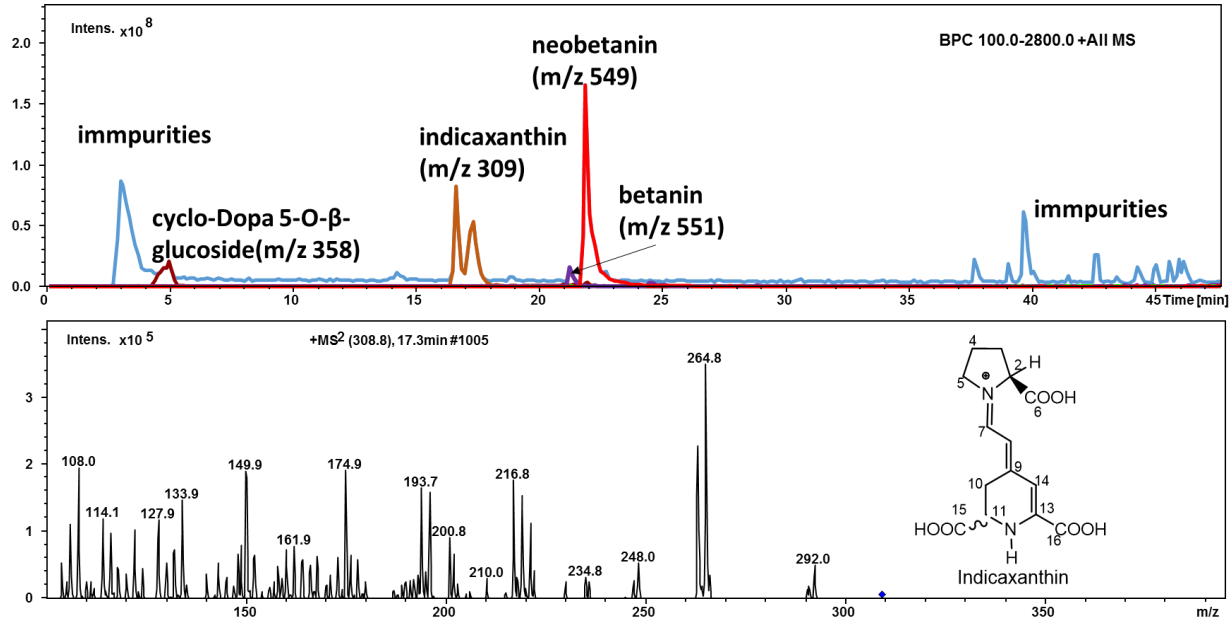


Figure 3-17: LC-ESI-MS chromatogram of synthesized indicaxanthin

3.2.4 Conclusions

This work opened a route for simple method for betaxanthins synthesis using chitin adsorbent for in-situ release of betalamic acid from simple step purification and hydrolyzation of betacyanins. In order to up-grade the method on larger scales, more experiments on multiple types of amino

acids will be required to optimize the condensation conditions and to enhance the reaction yields. The synthesized betaxanthins could also be further purified by all-liquid countercurrent chromatography (CCC) technique to obtain large amounts for bioassays and human intervention studies.

3.3 THE STUDIES OF *OPUNTIA DILLENII* PHYTOCHEMISTRY BY MEANS OF CCC/CPC

3.3.1 Aim and scope of this study

This work was undertaken to purify the betalains from *O. dillenii* by applying countercurrent chromatography and centrifugal partition chromatography (CCC/CPC) approaches. Both fresh and heated extracts were used to investigate the thermal degradation of the pigments, and the effects of their alteration on the resolution of separation. All the runs were performed using the same solvent system which was usually used for CCC separation of betalains before.[80,78] To the best of our knowledge, betalains were not separated by a CPC apparatus before. In addition, the semi-polar metabolites of the fruits such as "peptides-derivatives" were also studied by HPCCC for the first time. The crude extract was initially screened prior by LC-ESI-MS/MS to overview potential target pigments (see section 3.3.1.3a). After solvent extraction, C18 reversed phase resin purification processes were carried out to remove most of the sugars, pectins, amino acids, fruit acids and to pre-concentrate the pigments/flavonoids. The flow chart displayed in Figure 3-18 overviews the experimental steps done within this study for both pigments and non-pigment metabolites.

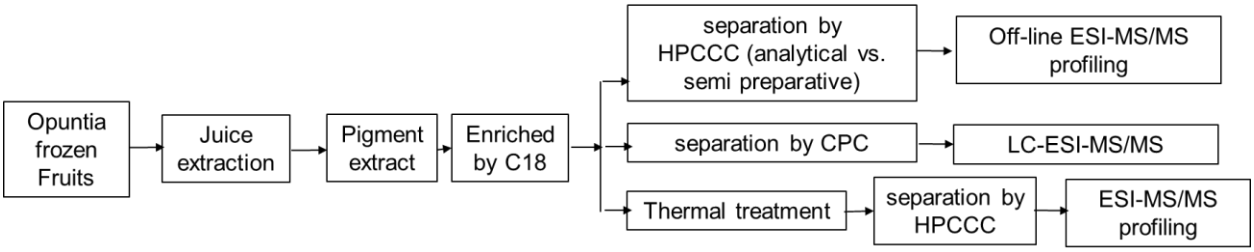


Figure 3-18: Overview on the experimental work within CCC/CPC study of *O. dillenii*

Table 3-8 shows all the CCC/CPC separations established together with their principle parameters.

Table 3-8: CCC/CPC separations performed within the study of *Opuntia stricta* fruits metabolites

Samples	CCC apparatus Coil volume	Solvent systems (v/v/v/v)	m_{sample} (mg)	$V_{injected}$ (ml)	Flow rate
C18 extract	Analytical HPCCC 22.5 ml	TBMe-n-BuOH-ACN-water (2:2:1:5, v/v/v/v, 0.7%TFA)	50	1.5	Elution: 0.5ml/ min Extrusion: 12ml/min
C18 extract	Preparative HPCCC 125 ml	TBMe–n-BuOH-ACN-water (2:2:1:5, v/v/v/v, 0.7%TFA)	500	5	Elution: 5ml/ min Extrusion: 10ml/min
C18 extract	Preparative CPC 200 ml	TBMe–n-BuOH-ACN-water (2:2:1:5, v/v/v/v, 0.7%TFA)	520	5	Elution: 5ml/ min Extrusion: 5ml/ min
Heated C18 extract	Preparative HPCCC 125ml	TBMe–n-BuOH-ACN-water (2:2:1:5, v/v/v/v, 0.7%TFA)	330	5	Elution: 4ml/ min Extrusion: 5ml/ min
non-pigmented compounds	Preparative HPCCC 125ml	EtOAc/iso-PrOH/water (25:1:25, v/v/v)	550	5	Elution: 5ml/ min Extrusion: 5ml/ min

3.3.2 CCC/CPC separation of enriched betalain pigments

3.3.2.1 The coupling of high performance CCC (HPCCC) with ESI-MS/MS in fractionation of C18 pigment extracts: analytical scale vs. preparative scale

a) ESI-MS injection profile of analytical HPCCC separation

In this section, the C18 reversed phase enriched pigment extract from *O. stricta dillenii* was separated for the first time on a HPCCC (model: spectrum DE). The combination of an analytical scale separation (coil volume 22.5 ml, maximum rotation speed of 1600 rpm) with the solvent system TBMe–n-BuOH–ACN–water TFA monitored by ESI-MS injection profiling approach was able to fractionate 50 mg of this extract. The pigments were nicely fractionated from non-pigment compounds, while several minor pigments that were not detectable in the crude extract were significantly concentrated into few fractions. Figure 3-19 shows the sequential injected vials (dried) and the reconstructed MS profile of this run, in which the injections were split into two parts due to high concentrations of the eluting components at early elution time.

The most intensive pigment betanin and its C15 epimer (**1**) was identified with $[M+H]^+$ *m/z* at 551 and the characteristic MS^2 fragment ion at *m/z* 389 (betanidin moiety) eluting at small

elution volume until tube 31. These pigments were followed by the malonyl derivative phyllocactin (15*S*/15*R*) (**2**) which was represented by *m/z* 637 and MS^2 at *m/z* 389. These initial fractions also contained a few degraded pigments namely decarboxy- and dehydrogenated betanins which appeared at low ion intensities, showing a low concentration compared to betanin. The strong ion signal at *m/z* 568 (**3**) with the fragmentations at *m/z* 549 and 387, indicated a neobetanin derivative. Herbach et al. (2005) suggested that this compound was hydroxy-neobetanin with the –OH group attached to the C14–C15 double bond (cf. Figure 2-11). It was suspected as an artifact generated during thermal treatment of *Opuntia* and red beet materials.[161]

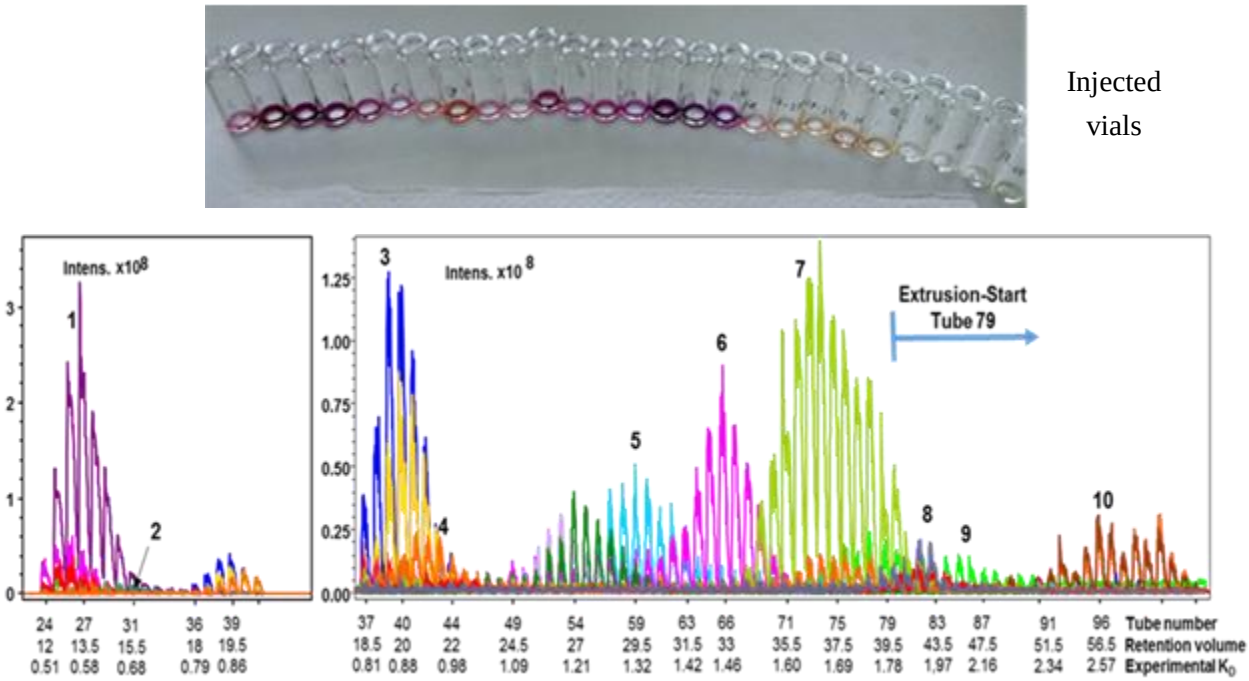


Figure 3-19: The sequential injected vials (up) and re-constructed ESI-MS injection profile of analytical scale HPCCC separation of *O. dillenii* C18 extract in pos. ionization mode (low)

Indicaxanthin (*m/z* at 309, **4**) was displayed as the only betaxanthin existing after the enrichment process and it was well separated from betanin during the analytical separation, despite the fact that it co-eluted with (**3**). As expected, the less polar aglycone betanidin with *m/z* at 389 (15*S*/15*R*, **6**), eluted between volume 31-35ml closely to the two dominant flavonoid glycosides, isorhamnetin-rutinoside (*m/z* 625 and MS^2 at 317, **7**) and kaempferol-rutinoside (*m/z* 595 and MS^2 at 287, **8**). The late elution of the betacyanin principle aglycone was caused by higher affinity to the stationary phase due to the loss of the polar glucose unit.

As it can be seen, the elution order of these pigments were identical to the observation in LC-ESI-MS/MS screening of the crude juice (section 3.1.4.2a), except for compound (**3**). The interestingly degraded hydroxyl structure appeared significantly in HPCCC separation but was

not detected in the LC-ESI-MS/MS of crude extract. Probably this minor compound was concentrated during HPCCC separation. In the case of betaxanthin, the addition of ion-pair reagent dramatically shifted (**4**) to the upper organic phase and kept the compound longer on the coil column compared to its immediate elution during the LC-MS analysis. The strong ion-paring effect of TFA on betaxanthins was already known from the previous HSCCC work on betalains, from which indicaxanthins eluted at increased elution volumes much later than betanin due to its interaction and stronger influence of TFA than glycosylated-betacyanins.[45]

The HPCCC resolved betanins and its derivatives resulted in several purified fractions with highly concentrated (15*S*/15*R*)-betanin. Additionally, the visualization of some unknown minor structures (**5**, **9**, **10**) (cf. Table 3-8) mainly present in the extrusion process (starting from tube 79) were of interest. These results suggested to scale-up this study by using the preparative coil column HPCCC under similar working conditions, solvent system, rotation in order to recover larger amounts of the target compounds, and to compare the reproducibility and separation capacity between the two experimental scales.

b) MS profile of preparative separation: the comparison between analytical (anal) vs. preparative (prep) run

The coupling of the preparative HPCCC with offline ESI-MS/MS injection monitoring of fractions was capable to fractionate and visualize by molecular weight 500 mg of C18 pre-purified pigment extract. Figure 3-20 shows the modified ESI-MS profile of this preparative separation.

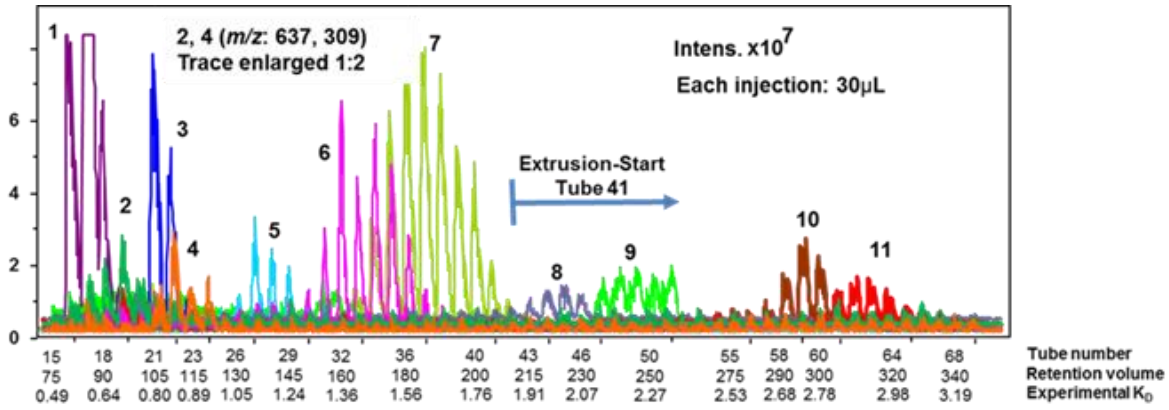


Figure 3-20: The re-constructed ESI-MS injection profile of preparative HPCCC separation of 500 mg C18 pre-purfied *O. dillenii* pigment extract (pos. ionization mode)

The elution orders of almost all of the compounds of interest were identical to that of analytical experiment; e.g., the betanin eluted initially after the break-through of solvents starting until tube 14, whereas most of the unknown structures were found later occurring during the extrusion

process. The comparison of selected ion traces from this run with that of analytical run showed that the most intensive signals of 15*S*/15*R* betanin (**1**) were detected with high ion yield in the initial fractions of both runs (anal: tube 24 - 30, prep: tube 16 - 18). Pigment (**1**) co-eluted with its precursor namely cyclo-dopa-5-O-*β*-glucoside (*m/z* 358, neutral loss Δ *m/z* 162) since they also occurred within these fractions; while the other precursor betalamic acid (*m/z* 212, in traces) was well separated from betanin and eluted lately from tube 46 to 50. Additionally, the common degraded products of betanin including neobetanin (*m/z* 549, MS^2 *m/z* 387), mono decarboxy betanin (**13**, *m/z* 507, MS^2 at *m/z* 345) and decarboxyl-neobetanin (*m/z* 505) were also detected at low ion intensity in these fractions. The co-existing of these compounds caused the distinct dark red color for the initial pigmented factions (tube 24 in analytical and tube 14 in preparative runs). Noticeably, these degraded compounds were not present in the LC-MS chromoatogram of crude extract (Table 3.2, section 3.1.4.2a); the appearances of these artifacts suggested that the process might induce pigment degradations, which is in agreement with the previous study of betalains using ion-pair HSCCC.[161,45,73]

The epimeric mixture of phyllocactins (15*S*/15*R*) was partly separated from 15*S*/15*R* betanin since the elution volume was slightly larger with about one or two fractions (anal: tube 26-32, prep: tube 16-21). The malonyl-substitution made these pigments less polar and kept them longer on the coil column compared to the non-acylated betanin structures. The intensive signals of hydroxy neobetanin were found close to (**2**) in both runs (anal: tube 36-44, prep: tube 21-22). This red derivative displaying stereoisomeric structure at C15 chiral center was well separated from betanins, although co-elution with the unknown signal at *m/z* 568 occurred.

As expected, the less polar aglycone betanidin/isobetanidin (**6**) occurred much lately in the HPCCC sequences (anal: from tube 62 - 69, prep: from tube 31 - 37) compared to the glycosidically bound betanins. These pigmented fractions also contained the more lipophilic structures of 15*S*/15*R* feruloyl-betanin (*m/z* 727, MS^2 *m/z* 389). This polar structure was supposed to be lampranthin II or gomphrenin III through the neutral loss of Δ *m/z* 176 representing for the cleavage of feruloyl unit, which had also been found in *O. ficus* before.[45] Surprisingly, the 15*S*/15*R* betanidin 5-O-*β*-sophoroside (*m/z* 713) was undetectable in both runs although it was concentrated in the study of chitin adsorption (section 3.3.1.3), and also identified previously in the HSCCC separation of *O. ficus* pigments. This result could indicate a very low concentration of betanidin 5-O-*β*-sophoroside in *O. dillenii* compared to *O. ficus*; and also confirm the high adsorption capacity of very polar betanin molecules such as di-glucosides to chitin compared to C18 resin. Although 15*S*/15*R* hylocerenins (*m/z* 695) is known as a typical cactus pigment, this compound was not recognized in both current separations which was similar to the phytochemistry of *O. ficus* extract studied by IP-HSCCC before.[45]

Additionally, the appearances of a degraded pyridine-derivative, 2,17-bidecarboxy-2,3-dehydro-neobetanin, (*m/z* 459, MS^2 at m/z 297) was found next to betanin in both runs; where the analytical experiment resolved the derivative from the pigment better than preparative experiment. Pyridine components have been identified in red beet and *O. ficus* as well.[162,45] The traces of eucomic acid (*m/z* 241), one of the typical organic acids found in *O. ficus*, was concentrated by HPCCC into few initial fractions due to its polar structure. Figure 3-21 displays the reconstructed selected single ion traces and corresponding fragmentation of several minor compounds identified in both separations.

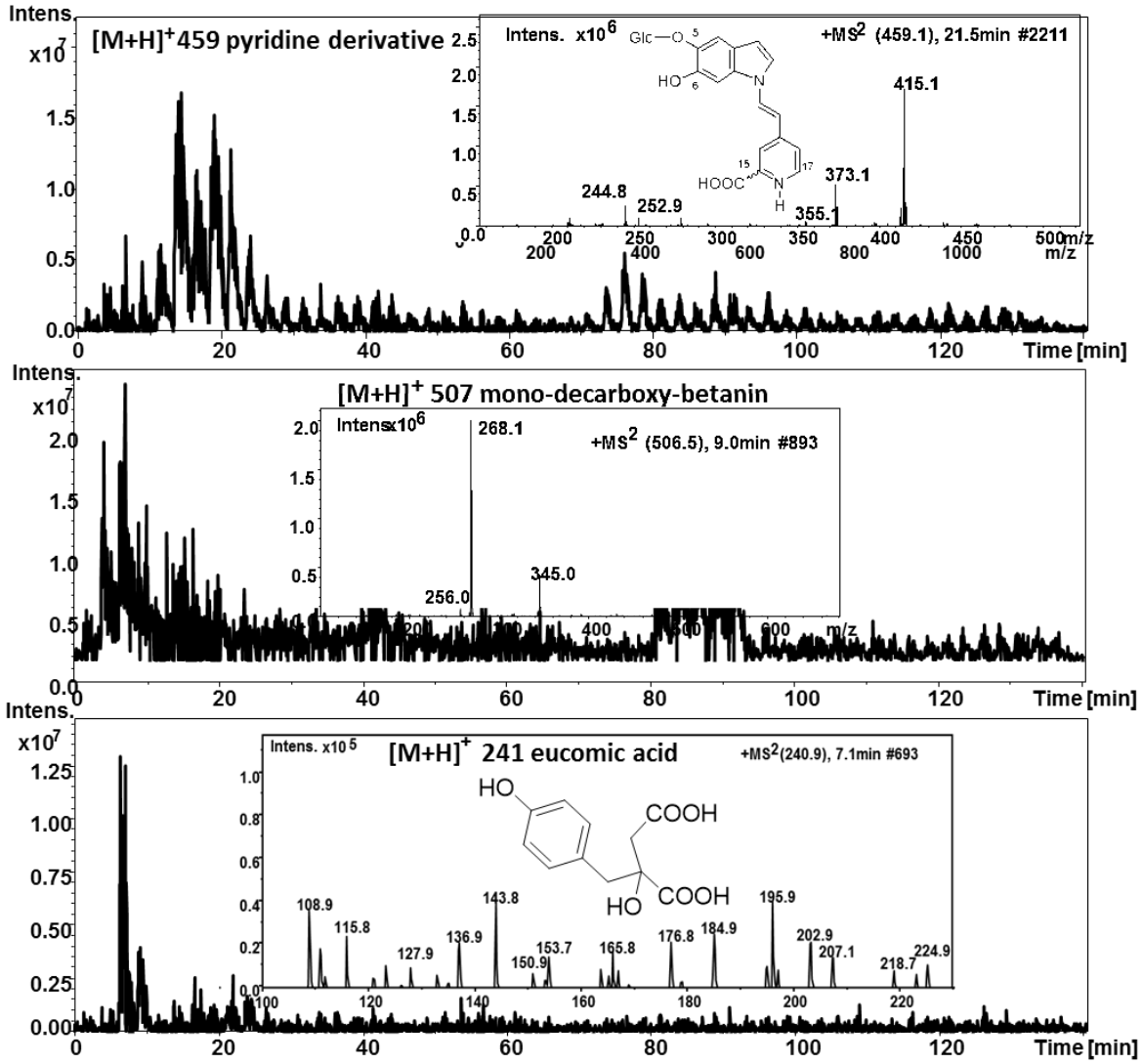


Figure 3-21: Selected ion traces of some compounds in the reconstructed preparative HPCCC ESI-MS/MS profile with MS^2 fragmentation of $[M+H]^+$ molecule ion signals

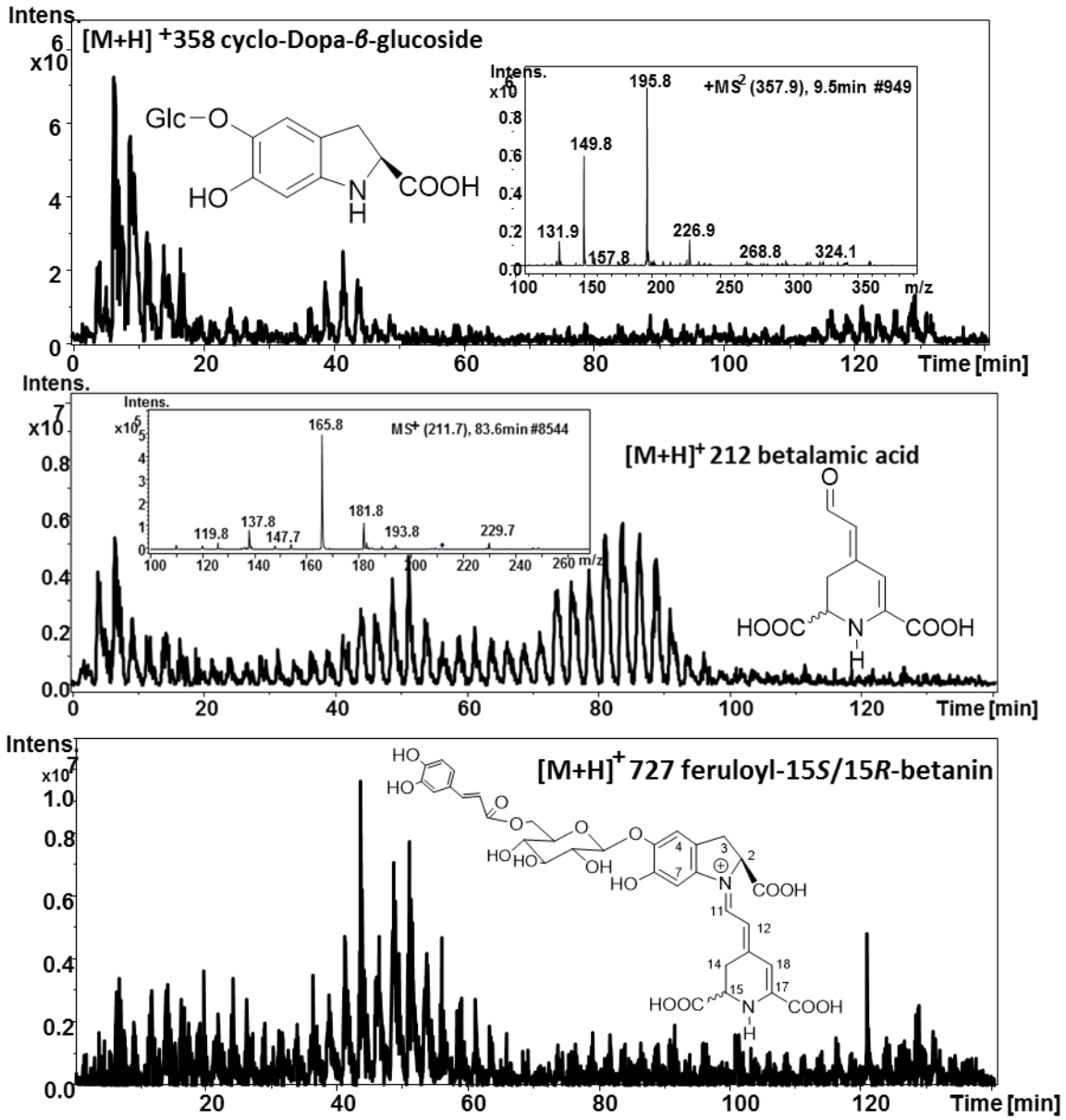


Figure 3-21 (cont): Selected ion traces of some compounds in the reconstructed preparative HPCCC ESI-MS/MS profile with MS^2 fragmentation of $[M+H]^+$ molecule ion signals

Looking closer into both HPCCC-ESI-MS injection profiles, there were fractions containing mainly betanin type compounds which appeared to be present in high purity (anal: tube 27-29, prep: tube 15-16). Nevertheless, these fractions consisted of 15*S*/15*R* betanin and also of their 6-O-glycosidic positional isomers gomphrenin I/isogomphrenin I, which were known to co-exist in the crude extracts. The HPCCC frationation of betanin from these 6-O-glycosidic isomers cannot be visualized by a ESI-MS profile approach since all these compounds are isobars with the same $[M+H]^+$ ion trace at *m/z* 551 and MS^2 fragment at *m/z* 389. The IP-HSCCC separation of 15*S*/15*R* betanin and 15*S*/15*R* gomphrenin I from *O. ficus* extract was detected by LC-MS/MS

approach using a second chromatographic dimension with the LC-C18 phase. In addition, that LC-monitoring experiment also confirmed the partly fractionation of 15*S* from 15*R* betanins, in which the 15*R* isomer eluted slightly earlier than its counterpart. The prior elution of 15*R* was inversed from the elution order observed in the C18 RP-LC-MS chromatogram.[45] The successful HPCCC fractionation of 15*S* and 15*R* isomers with identical molecular weight (isobars) and the various mono decarboxybetanin degradation products cannot be evaluated by the ESI-MS injection profile in case the elution area overlap. There were HSCCC separations of cacti fruits pigments such as *O. ficus* and *Hylocereus polyrhizus* done before; where a partial isolation of non-acylated betanins from acylated betanin (e.g., betanin isolated from phyllocacins and hylocerenins) was achieved. However, the isolation of their respective diastereomers 15*S* and 15*R* has not been achieved despite the addition of the much stronger perfluorinated carboxylic acid as heptafluoro glutyric acid HFBA.[73]

The non-pigment related compounds eluted mostly in the extrusion-mode of both runs consisting of many unknown structures which were recovered with more impurities. The not-identified component (**5**) (*m/z* 689) occurred in a larger range of fractions from tube 49 to 63 (anal) and tube 24-29 (prep), and it partly co-eluted with the minor signals at *m/z* 538 and *m/z* 395 (cf. Table 3-8). These two unknown signals were partly separated from indicaxanthin in the analytical run (tube 51-59) but they co-eluted completely in the preparative run (tube 25-29). The most concentrated flavonoid glycoside, isorhamnetin rutinoside (**7**), was nicely separated from betanidin (**6**) but also distributed in a broad range of fractions (anal: tube 68-80, prep: tube 32-40). (**7**) produced very strong ion signal intensity and overlayed the co-elution of the unknown signal at *m/z* 647. The second abundant flavonoid glycoside, kaempferol-rutinoside (**8**) occurred closely to the isorhamnetins (fraction 81-84 (anal), fraction 40-45 (prep) at lower intensity. Despite the co-elution with other unknown compounds, several fractions of these two flavonoid glycosides seem to be pure and could be directly used for structural elucidation by NMR. The presence of these flavonoid glycosides in *Opuntia spp.* are varying depending on species and had been frequently reported. Their strong potential bioactivities and health effects were well discussed.[146] Surprisingly, none of the alkaloid related pseudomolecular $[M+H]^+$ ions at *m/z* 484, 498, 406 and 288) in *O. ficus indica* fruits found by previous HSCCC separation were detected in the *O. stricta dillenii* fruits. In addition, the most lipophilic compound eluted at the end of the extrusion process displaying at $[M+H]^+$ *m/z* 620 (**10**) and $[M+Na]^+$ *m/z* 642, which have not been described in any studies on *Opuntia. spp* before and suggesting for the cyclic peptides.

In general, the elution order of the principle compounds in analytical run were identical to that of preparative run and LC-MS chromatogram of crude extract, except for some differences which were due to the effect of TFA.

c) Comparison of partition coefficient K_D values between analytical and preparative runs

In order to compare the productivity of this HPCCC spectrum between two experimental runs, the actual partition coefficient K_D value of the target compounds were calculated based on their retention volumes according to the equation 3-2 below:[59]

$$K = \frac{V_R - V_{SF}}{V_{C-V_{SF}}} \qquad \text{(Eq. 3-3)}$$

V_R is the actual retention volume of a specific compound eluting in each run, V_{SF} is the volume of mobile phase passing through the coil for reaching equilibrium, V_C is the total coil volume (analytical 22.5 ml and preparative 125 ml). In this study, the retention of stationary phase S_f were 75.5% and 73% for both runs, respectively. For each specific compound, both the peak start and end were used to calculate the K_D range using the elution volume approach.

Despite the small difference between the retention time S_f of two experiments, the corresponding K_D values showed significant variations (data not shown). After the extra column volume (V_{ext} or the "dead" volumes) consisting of flying leads, connecting line between pump and fraction collector etc. were taken into account according to Costa et al. (2016), the K_D values between two runs were reasonably identical. The K_D values adjusted by the dead volume were illustrated in Table 3-9. As displayed, most of the K_D values of analytical runs had higher values than the preparative one, except for compounds **1**, **8** and **10**. The largest differences between two runs exhibited within compounds **6**, **7**, **8** and **10**, in which the K_D of anal run indicated broader elution or lower resolution than that of the prep run.

Table 3-9: Summary of target compounds and the K_D values of analytical and preparative HPCCC separations after the adjustment

No	*m/z*	MS^n	Compounds	K_D	
				Anal	Prep
1	551	389	15*S*/15*R* betanin 15*S*/15*R* gomphrenin	0.51-0.76	0.54-0.64
2	637	551, 389	15*S*/15*R* phyllocatin	0.56-0.81	0.54-0.80
3	568	549, 387	hydroxyl-neobetanin	0.79-0.98	0.79-0.85
4	309	263	indicaxanthin	0.86-1.0	0.79-0.95
5	689	645, 603	Not ident.	1.19-1.44	1.05-1.20
6	389	345	15*S*/15*R* betanidin	1.39-1.56	1.25-1.61
7	625	317, 257, 479	isorhamnetin-rutinoside	1.56-1.93	1.40-1.87

8	595	449, 287	kaempferol-rutinoside	1.79-1.98	1.91-2.07
9	506	268	Not ident.	-	2.12-2.30
10	620	602, 342	Not ident.	2.35-2.91	2.63-2.78
12	727	389	15*S*/15*R* feruloyl-betanin	1.35-1.60	1.25-1.56
13	507	345	mono decarboxy-betanins	0.51-0.63	0.49-0.59
14	358	196, 150	cyclo-dopa 5-O-*β*-glucoside	-	-

This V_{ext} adjustment was applied in the scale-up study on the extract of *Schinus terebinthifolius* berries purification using this HPCCC instrument before, resulting in roughly similar K_D values between analytical and preparative runs. The authors suggested the subtraction of 4.5 ml for analytical column and 7 ml for preparative column as "dead" volumes for this HPCCC setup.[163] It was announced that the extra-coil volumes could cause the offset in actual K_D calculation since these volumes might increase the real elution volume of the eluates and its elution times, and lead to the inaccurate prediction/calculation, especially for the small analytical coil volume.[58] In this study, the effect of dead volume in analytical scale was significant (more than 20% of coil volume) while it is almost neglectable in the preparative scale (less than 6%). The differences found in the K_D value calculation before and after adjustment confirmed the importance of extra column volume in CCC approaches.

Likewise, the volume of sample injected also causes variation in K_D calculation, particularly in the analytical separation, because the large volume of sample introduced into the system could shift the actual retention volume to half of the sample loop (equal to the portion of mobile phase in the sample solution). It has been suggested that $5\%V_C$ is the suitable sample volume which does not affect the hydrodynamic equilibrium under standard operation; whereas the sample concentration of 100 mg/ml is normally used for scale-up studies of CCC.[164] The sample concentrations in this study were 50 mg/1.5 ml for analytical and 500 mg/5 ml for preparative runs, which accounted for 6.7% and 4% of total coil volumes, respectively. This volume might also contribute to K_D variation between two observed HPCCC runs. Additionally, the bleeding of stationary phase could solely decrease the S_f of the system, particularly when the sample has emulsification effects to the solvent system. This should be taken into account for accurate K_D value calculation. Conway et al. (2005) introduced the method to involve these leaking volumes in K_D calculation.[58]

d) The comparison of the resolution $R_{i,j}$

In CCC approaches, the resolution R between two peaks (or two compounds) is calculated by the ratio between the difference of retention volumes and the total base width of these peaks (Figure 3-22) according to equation 3-3:

$$R_{i,j} = \frac{2(V_{Rj} - V_{Ri})}{W_i + W_j} \qquad \text{(Eq. 3-3)}$$

This value illustrates the separation power between two peaks, in which the higher value of $R_{i,j}$ shows the better fractionation. For instance, the $R_{i,j}$ value lower than 1 represents an overlapping area larger than 2.3% of peak areas, or these two compounds co-elute at more than 2 out of 6 baseline portions corresponding to normal distribution.[165]

Vieira et al. (2015) adapted the method to calculate the $R_{i,j}$ value from a modified ESI-MS profile, from which the base width of selected peaks (ion traces) are represented by total elution volumes of all the fractions containing that peaks. The resolution of several pigments and non-pigments compounds were calculated for both analytical and preparative experiments and are displayed in Table 3-10

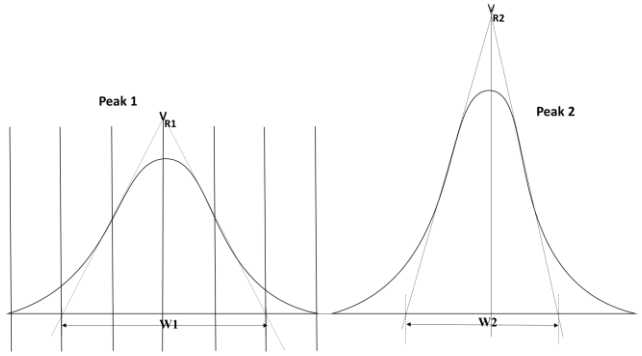


Figure 3-22: Peak resolution in the correlation with normal distribution

Table 3-10: The resolution of several selected peaks of both analytical and preparative HPCCC experiments

R	$R_{1,2}$	$R_{2,3}$	$R_{3,4}$	$R_{4,5}$	$R_{5,6}$	$R_{6,7}$	$R_{7,8}$	$R_{1,4}$	$R_{1,6}$	$R_{1,3}$	$R_{1,12}$	$R_{1,13}$
Analytical	0.25	0.75	0.25	1.56	0.64	1,00	0.64	1.31	3.6	1.0	3.0	0.33
Preparative	0.33	0.25	0.33	0.625	1.0	0.44	1.0	1.57	1.65	1.3	3.2	0.33

As it can be seen, the resolution values were very different between the two performed HPCCC separations, from which the analytical values appeared to be lower in most of the selected pairs than that of the preparative (with several exceptions). For instance, the separation of betanin and phyllocactin on the large-scale was 1.3 times better than the small-scale run, ($R_{1,2}$, 0.25 vs. 0.33); a similar case was found for phyllocactin and indicaxanthin ($R_{3,4}$, 0.25 vs. 0.33). These results

indicated a better resolution power for the large-scale separation applied to this mixture of pigments and non-pigmented substances. There was a study showing the positive correlation between coil volume and resolution, in which the larger coil volume might allow better mass transfer rate among the solutes with stationary and mobile phase, and lead to a higher resolution within the scale-up factor from 100 up to 1000. The increase of bore size and length of the CCC coil column enable better separation power due to the larger interfacial areas and make it capable to handle high sample load.[166,167,168]

However, a closer look into the data illustrated a better separation power for the analytical scale observing some specific pairs compared to the preparative run. For instances, betanin and betanidin were fractionated two times better in the analytical run than the preparative run ($R_{1,6}$, 3.6 vs. 1.65); a similar result was seen in the pair of phyllocactin and hydroxy-neobetanin ($R_{2,3}$, 0.75 vs. 0.25). These results were in good agreement with the report of Berthod et al. (2008) that the smaller coil volume with small bore tubling enhance the resolution.[169] On the other hand, the low sample load (500 mg/5ml vs. 50 mg/1.5ml) might reduce the sample emulsification and prevent the broadening or overlaping of the peaks; and it could contribute to the high peak resolution of the small-scale HPCCC operation.[164]

In brief, the adjustment of these extra coil volumes on the used HPCCC set-up resulted in comparable K_D values observed for all target compounds between two runs; that confirmed the importance of 'dead' volumes in K_D evaluation within scale-up studies. In the CCC approach, the analytical trials are often done prior for solvent system selection, optimization of the operating parameters such as sample loading amount and volume, flow rate, etc. The small-scale operation is efficient in terms of time saving, solvents and operating costs in order to achieve the optimized parameters, especially when new samples are separated for the first time in a CCC configuration. These smaller scale runs are also applied numerously in the scale-up studies of natural products, in which the productivity and resolution are of high interest.[170,168] The application of both analytical and preparative scales for *O. dillenii* extract under the constant operation conditions (temperature, rotation speed and corresponding g-force) indicated that this typical solvent system TBMe/n-BuOH/ACN/water TFA was applicable to fractionate the complex metabolite of enriched pigments extract, resulting in the fractionation of pigments from non-pigment compounds. This approach was shown to be efficient for the high sugar containing betalainic plants with the capability to be scaled-up for high pigment recovery with comparable resolution power.

3.3.2.2 Study on thermal degradation of O. dillenii pigments: HPCCC-ESI-MS sequential injection profile of 'heated' pigments separated by preparative-HPCCC

a) Pigment degradation: the effects of thermal treated pigments on the elution order within ion-pair HPCCC

The previous used C18 enriched extract was treated at 85^{0}C in a sealed reactive vial before a preparative HPCCC separation. The eluted fractions were also monitored by ESI mass-profiling approach in order to compare the induced metabolites of the pigment extract before and after thermal treatment. The ESI-MS injection profile is illustrated in Figure 3-23.

As can be seen, the elution order of target compounds on this HPCCC run were identical to that of the non-heated sample (cf. Figure 3-20) although the volume of breakthrough solvent was slightly larger (until tube 18 vs. tube 15). The pigments were recovered mainly in the elution-mode while most of the unknown structures eluted lately during the extrusion. The betanin and its epimers concentrated in about three initial fractions (tube 18-20) and partly mixed with 15*S*/15*R* phyllocactin as expected. Interestingly, higher ratio of ion intensity between phyllocactin and betanin was recognized in comparision to previous runs (Figure 3-20), which might indicate the specific thermal resistance of this malonyl acylated betanin.

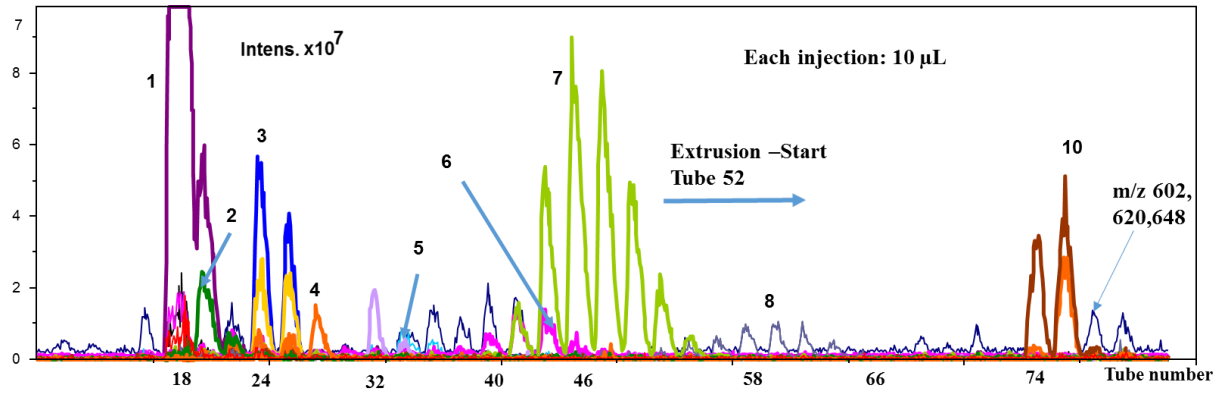


Figure 3-23: ESI- MS injection profile of thermal treated C18 enriched *O. dillenii* extract fractionated by HPCCC (pos. ionization mode)

Figure 3-24 illustrates the ESI-MS profile differences of fractionation of degraded pigments (selected ion traces) between heated and non-heated sample from two HPCCC separations. Thermal treatment is known to cause structural alterations in betalain.

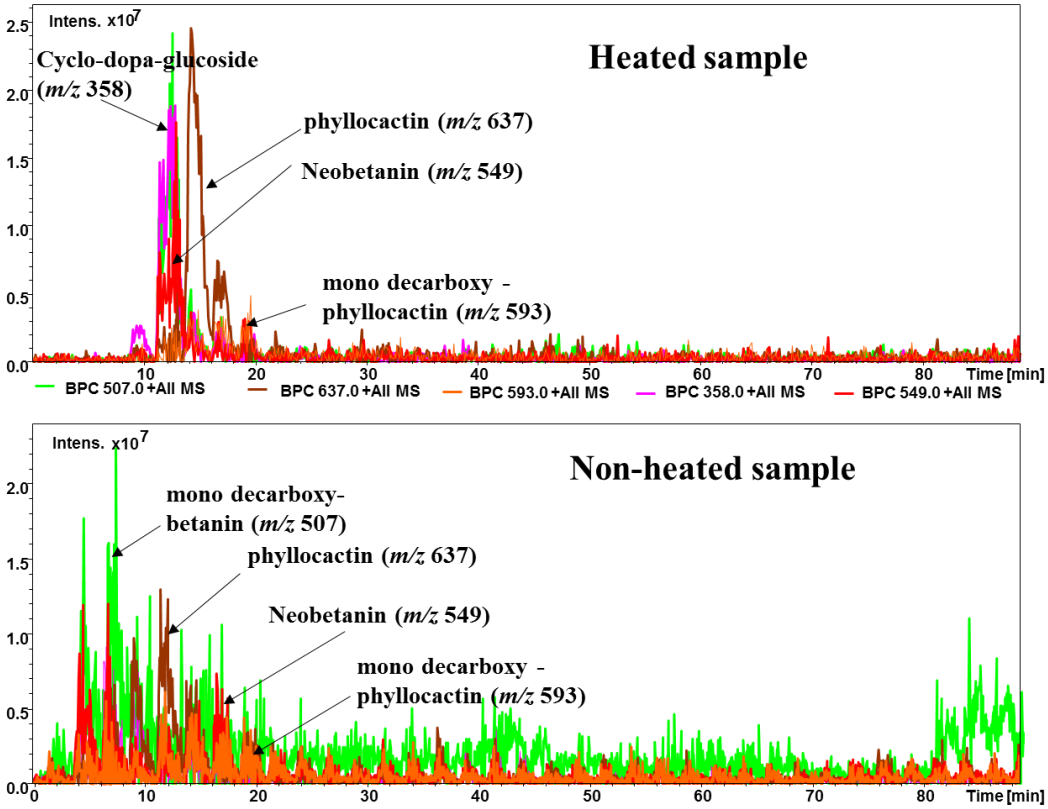


Figure 3-24: Elution of degraded betanin products and phyllocactin from non-heated and heated sample separated by HPCCC and monitored by ESI- MS injection

The intensive red color degraded products, such as neobetanin and mono decarboxy-betanin (**13**), were detected within several early eluting fractions together with betanin. This co-elution was found identical to the preparative fractionation of non-heated pigment extract, except the remarkable induced ratio of cyclo-dopa-glucoside and the slightly increase of decarboxybetanin due to heating effects. Similarly, the signal of neobetanin also occurred within the same range ESI-MS profile fraction and was seen in increased intensity compared to betanin. Since the red color decarboxybetanin and neobetanin naturally present in very low concentration within betalain producing plants, the majority of these artifacts are produced under extreme conditions of temperature or pH.[171,145,40] Their higher ratios found in this thermal treated sample confirmed the decarboxylation and dehydrogenation effects at high temparature. Surprisingly, these two degraded pigments were partly fractionated from phyllocactin although they were completely mixed in the non-heated sample separation (Figure 3-24). The decarboxy-neobetanin (at *m/z* 505 and MS^2 at *m/z* 345) was also found in traces which was in agreement with the results of Herbach et al. (2005).

Additionally, a very low ion intensity signal of decarboxy-phyllocactin, which displayed at *m/z* 593 (MS^2 at *m/z* 549, 345) was detected together with these betanin thermal artifacts; whereas the

degradation product of feruloyl-betanin was not found. This degraded product of phyllocactin was identified in the *Hylocereus* heated extract before.[135] The low intensity of this degraded malonyl acylated betanin in combination with the enhanced ratio of phyllocactin/betanin confirmed that pigments of *O. dillenii's* are highly heat resistant in a stabilizing mixture. That thermal stability is promoted by the acidic nature of the cultivar due to its high dose of fruit acids (pH 3-4), and the acylated structure of phyllocactin was also known for heat resistance.[171] This result was identical to the previously published study on the stabilites of cactus pigments under processing.[18] On the other hand, there were signals of betacyanin precursors betalamic acid (*m/z* 212) next to cyclo-dopa 5-O-glucoside (**14**, *m/z* 358) eluting early in the ESI-MS profile sequence. These two primary structures were also recognized in both HPCCC separations of non-heated sample (analytical and preparative experiment) although they were not detected in crude pigment extract. These precusors might be released through the hydrolytic cleavage of betacyanin aldimine bonds during thermal treatment or under drastic acidic conditions.[172] This was found to correspond to the reduction of betanin and betanidin ion intensities in the ESI-MS profile compared to previous HPCCC runs. These results could support the study of Herbach et al. (2006) in which betanidin is less heat stable than its glycosidic derivatives.[127] Likewise, the intensity of indicaxanthin (**4**) was found significantly reduced after this heating experiment. That is relevant as low stablility of betaxanthins compared to betacyanins under high temperatures was reported before. Figure 3-25 shows ion traces of several minor-degraded-structures released from the thermal treatment monitored from HPCCC experiment.

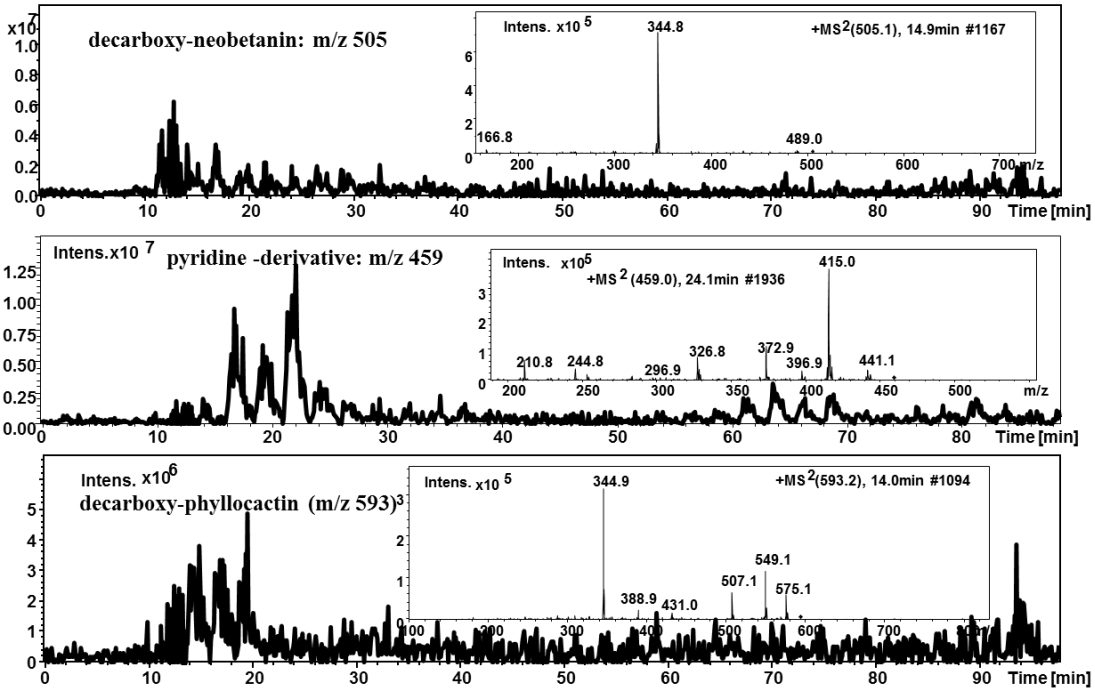


Figure 3-25: Selected single ion traces of some degraded pigments derived by thermal treatment and fractionated by HPCCC and ESI-MS injection

The signal of pyridine-derivative (*m/z* 459, MS^2 415) was also found identical to the non-heated experiment whereas eucomic acid disappeared due to its potential instability under high temperature (cf. Figure 3-21). There are more degraded products known from thermal treatment of purified betanin and phyllocactin solutions which were reported previously; however, these components were hardly detected in this part of study.[162,127] The contribution of the fruits matrices on betalain stabilization has been revealed, in which the natural constitutents, such as sugars, pectic substances and antioxidants as ascorbic acid and organic acids could protect the pigments from degradation reactions. Additionally, the purified pigments were found more susceptible to thermal treatments, and processing could generate more complex alterations in comparison with complete extracts with matrice.[161]

The isomerization of betalains under thermal treatment was intensively discussed; that reaction causes changes in the epimer ratios but not the visual color of the pigments. For instance, the isomerization of purified phyllocactin induced the ratio of isophyllocactin/phyllocactin; whereas purified betanin showed no difference in stereoisomer ratios due to the instability of isobetanin compared to betanin under heating was reported before.[173,40,127] Despite the fact that this heat experiment might enhance these epimer conversions, the ESI mass-profiling approach is unable to show the changes in ratios, as well as the fractionation possibility of of the betanin/isobetanin from gomphrenin/isogromphenin due to the identical molecular weight $[M+H]^+$ at *m/z* 551 and MS^2 at *m/z* 389.

Surprisingly, the series of unknown components recorded at m/z 602, 620, 648 (**9**, **11**), which were well detected in the extrusion-mode of previous separations, were hardly found in this heated experiment. These signals displayed low intensity within crude juice and were sugested as minor components present in the C18 enriched extract.

b) K_D calculation: The effects of thermal treatment on elution volumes of target compounds

The K_D values of interested compounds were calculated similarly to the previous experiment (cf. section 3.3.2.1d) in order to study the effects of thermal artifacts on HPCCC separation. The results are presented in Table 3-11. The distribution values showed some differences from that of the non-heated samples, particularly in the pigments such as betanin, betanidin and feruloyl-betanin. The different flow rates (4.0 ml/min vs. 5.0 ml/min) might cause the variation in solvent equilibrium between two preparative runs and enhance the retention S_f (80% vs. 78.6%). This was in agreement with the report of Du et al. (1999) on the reverse correlation between flow rate of mobile phase and retention of stationary phase.[61] Additionally, the changes in sample's metabolites under heating, the reduction of sample concentration (330 mg vs. 500 mg) might also affect the elution volumes. These reasons in combination resulted in the small increase in volume

of breakthough solvent, distinct carry-over of stationary phase that led to the very small differences in K_D values between two HPCCC preparative runs (cf. Table 3-9 and 3-11)

Table 3-11: K_D values of target compounds eluted from HPCCC separation of heated extract

m/z	551	637	568	309	689	389	625	595	620	727	549	358
K_D	0.47-0.55	0.55-0.63	0.71-0.79	0.79-0.87	1.03-1.19	1.35-1.59	1.43-1.83	1.99-2.15	2.71-2.79	1.43-1.59	0.47-0.55	0.47-0.55

c) $R_{i,j}$ calculation: The effects of thermal treatment on the resolution of target compounds

The resolution of target compounds was calculated and is displayed in Table 3-12. As it can be seen, these $R_{i,j}$ values were generally higher in comparison with that of the non-heated HPCCC experiment (Table 3-10), presenting a better fractionation in this run. For instances, the resolution between two principle pigments betanin and phyllocactin was doubled ($R_{1,2}$, 0.67 vs. 0.33) which could be due to the decrease of betanin concentration under heating. Similar rise in resolution was found in the pairs of hydroxy-neobetanin and betaxanthin ($R_{3,4}$, 0.67 vs. 0.33) while that of phyllocactin and hydroxyl-neobetanin reached five times ($R_{2,3}$, 1.33 vs. 0.25). The highest resolution occured within betanin and feruloyl-betanin ($R_{1,12,}$ 6.25 vs. 3.2) followed by that of betanin and betanidin ($R_{1,6,}$ 4.8 vs.1.65), in which this experiment with heat treated pigments displayed superior separation power than the separation of genuine pigments. Nevertheless, the betanidin and isorhamnetin rutinoside were poorly resolved in this run and exhibited similar $R_{6,7}$ of 0.44. As discussed above, these results confirmed that the decrease in flow rate and sample concentration enhanced fractionation power under constant HPCCC operating conditions. Noticeably, thermal treatment causes changes in metabolite in the samples by generating artifacts, these alterations not only interfered with the purity of collected fractions, but they might have also interfer with the equilibrium and resolution of HPCCC experiment. For example, betanin and its artifact cyclo-dopa 5-O-*β*-glucoside were not well fractionated and co-existed in the initial fraction ($R_{1,14}$, 0.5).

Table 3-12: $R_{i,j}$ values of target compounds separated from heated pigment extracts by HPCCC

R	$R_{1,2}$	$R_{2,3}$	$R_{3,4}$	$R_{4,5}$	$R_{5,6}$	$R_{6,7}$	$R_{7,8}$	$R_{1,4}$	$R_{1,6}$	$R_{1,3}$	$R_{1,12}$	$R_{1,14}$
	0.67	1.30	0.67	1.75	1.50	0,44	1.38	2.67	4.80	2.00	6.25	0.50

The studies on betalain thermal stabilities are of interest since most of the commercial betalain products require processing before consumption. This present study was initiated to confirm the changes in the metabolites of *O. dillenii* extract before and after the thermal treatment and the heat resistance of these pigments. That superior properties of *O. dillenii* color stabilities could be a promising candidate for replacement of red beets as betalain source. The approach by HPCCC was unable to resolve betanins and its thermal degraded derivatives, although it exploited better separation capacity than the non-heated experiment. The stronger perfluoro carbocylic acid such as HFPA might be needed for higher ion-pair capacity and better separation of these degraded structures. Nevertheless, these acids are harmful to environment and are not generaly considered as 'food grade' chemicals; which make the isolated pigments not applicable for bioassay. In terms of HPCCC purification, the reduction in flow rate and amount of sample injected could enhance the resolution of target compounds. However, the lower flow rate will prolong the operation time while reduced sample concentration will minimize the productivity, which would always be a controversal issue of CCC.

The combination of HPCCC with ESI-MS/MS injection profiling creates a single molecular weight data file of all ionizable compounds present in every injected fraction. This profile allowes the quick selection of target ion traces (or components) for directed identification by LC-MS or NMR, or to apply further purification steps. However, this approach could not distinguish the isomeric structures such as the decarboxy betanins consisting of distinct cleavage of a carboxyl group at C2, C15 and C17. These epimers are only identified by their different retention times on C18 analytical HPLC columns.

3.3.2.3 Preparative separation of C18 enriched pigment by centrifugal partition chromatography (CPC) monitored by off-line LC-ESI-MS/MS analysis

Up to now, countercurrent chromatography (CCC) has been used commonly to fractionate betalains from many plant sources. This was the first time that betalains were separated by a CPC apparatus on lab-scale using the standard TBMe/n-BuOH/ACN/water TFA solvent system. This was aimed to isolate *O. dillenii* pigments by CPC under roughly identical operating conditions as HPCCC, and to compare their separation powers throught actual K_D and $R_{i,j}$ values. Since the ESI-MS injection profile showed limitation in visualizing the fractionation of epimers, ESI-LC-MS analysis was used for the second chromatographic dimension separating 15*S* and 15*R* epimers.

The machine was operated at medium speed of 1000 rpm resulting in a retention S_f of 72% (flowrate 5 ml/min), and fractionated 520 mg of C18 enriched extract. After the breakthrough of mobile phase until tube 13, the pigments started eluting from fraction 14 (identical to that of

preparative HPCCC). The LC-ESI-MS/MS analyses of the intensively pigmented fractions (from tube 15 to tube 32) displayed the complex metabolites of betanins and its derivatives. As expected from previous separations, betanin and its stereoisomers including 15*S*/15*R* epimers and 6-O-*β*-glucosidic positional isomers appeared very early in the CPC fraction sequence, particularly concentrated in tubes 18 to 21. Likewise, the neobetanin, mono decarboxy-betanin, mono decarboxy-neobetanin were clearly detected from these initial fractions of 15-18 together with the intensive appearance of cyclo-dopa-5-O-glucoside. The prompt elutions of these degraded pigments were identical with that from HPCCC experiments (section 3.3.2.1), from which they were supposed to be generated during processing. Surprisingly, betanin (**1**) were better separated from these alterated products in comparison with the HPCCC where these pigments completely co-eluted. Closer examination of the chromatograms of these pigmented fractions indicated that the separation of betanin and isobetanin was partly successful. This was in agreement with the HSCCC fractionation of *O. ficus* pigments before in which these C15 isomers were partly resolved; that study also described the early elution of isobetain compared to betanin. From fractions 23 to 32, four peaks were detected exhibiting the same *m/z* at 551 and generating MS^2 at 389, which were identifed as 15*S*/15*R*-betanin and15*S*/15*R*-gomphrenin.The partial separation of these 5-O-*β* and 6-O-*β*-glycosidic derivatives was achieved due to a later elution of gomphrenin than betanin. The partly fractionation of these four pigment isomers is illustrated in Figure 3-26. These fractions also contained the second abundant pigments phyllocaction/isophyllocactin in lower concentration.

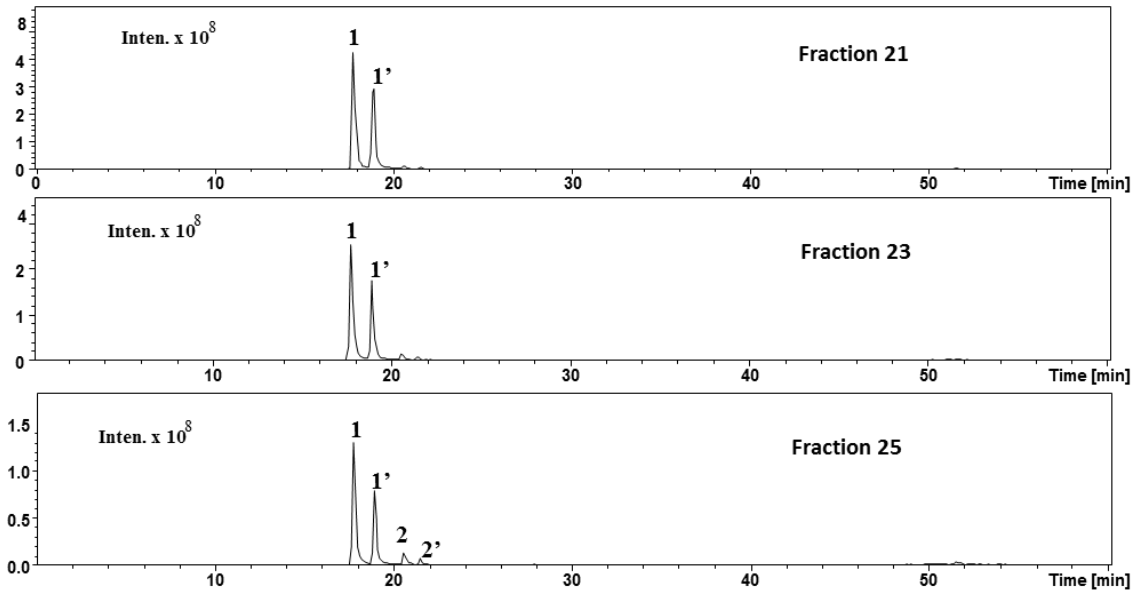


Figure 3-26: The modified LC-MS/MS chromatogram of fractions containing betanin/isobetanin (1/1') and gomphrenin/isogomphrenin (2/2')

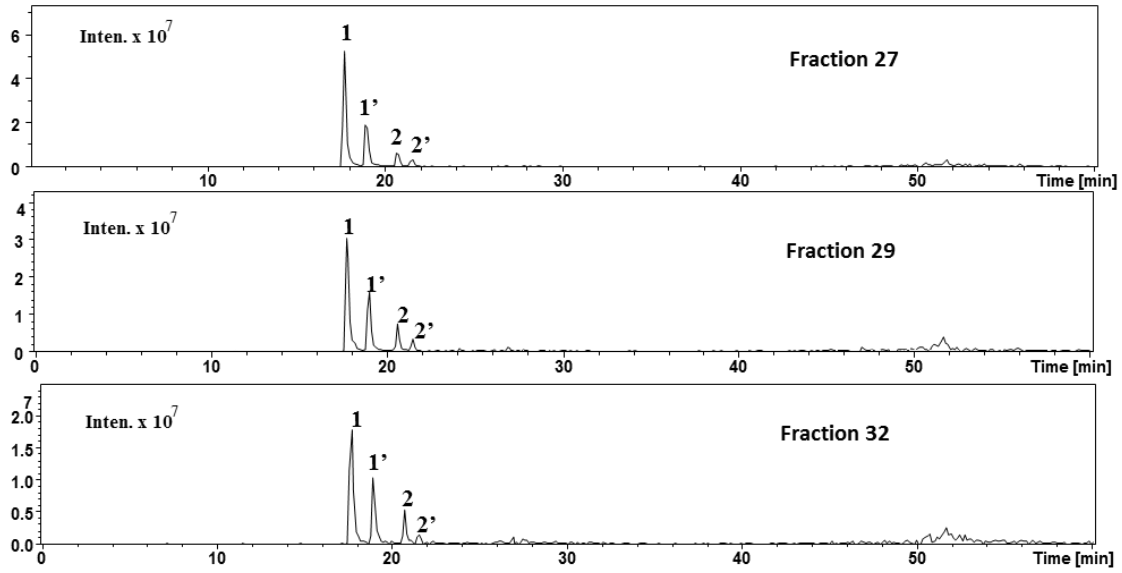


Figure 3-26 (cont): The modified LC-MS/MS chromatogram of fractions containing betanin/isobetanin (1/1') and gomphrenin/isogomphrenin (2/2')

Figure 3-27 illustrates the distribution of pigments and their derivatives within the collected fractions based on their peak areas elucidated from ESI-LC-MS chromatograms of each fractions. Similarly, the distribution of the non-pigmented compounds were also elucidated and is shown in Figure 3-28.

The highly polar betanidin 5-O-*β*-sophorosides (15*S*/15*R*) eluted very early in the sequence (fraction 15-16) although they were hardly detected by the MS profiling approaches within previous HPCCC runs. This minor pigment was known as the most polar glucosyl-betanin naturally existing in cacti fruits of H*ylocereus polyrizhus* and *O. ficus*.[45,54] Traces of feruloyl-betanin (**13**), the most lipophilic betacyanin found in this cultivar, was recognized in a wide range of fractions from 69 to 78. The late elution of this pigment due to its strong affinity to the organic phase was found to be identical to previous HPCCC separations. Likewise, the aglycone betanidin was also detected within this range of fractions, that was relevant to its highly hydrophobic structure. The stronger effects of TFA on betaxanthins resulted in longer retention of indicaxanthin from fractions 32 to 50 and was in accordance with the HPCCC separations

As it can be seen, most of the lipophilic structures including betanidin, two flavonoids glycosides and several unknown structures (**9**, **10**) were co-eluted at the end of the CPC sequence within the extrusion-mode. Surprisingly, there was no separation of indicaxanthin and the unknown structure **5** (*m/z* 689) although they were partly fractionated by HPCCC experiments.

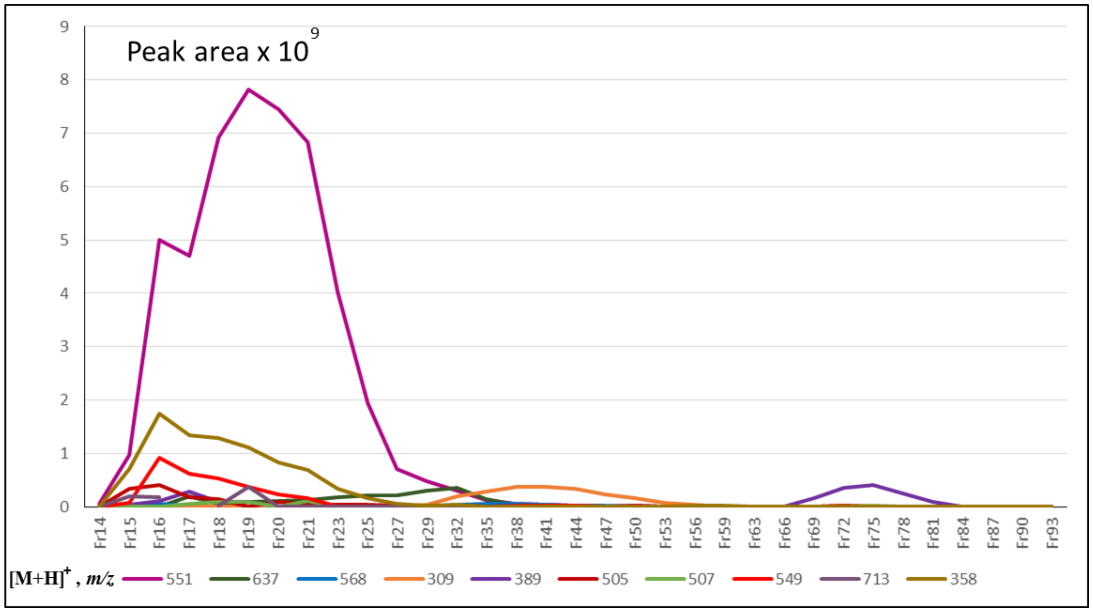


Figure 3-27: The distribution of *O. dillenii* pigments eluting from CPC separation based on the peak areas elucidated from LC-ESI-MS analyses

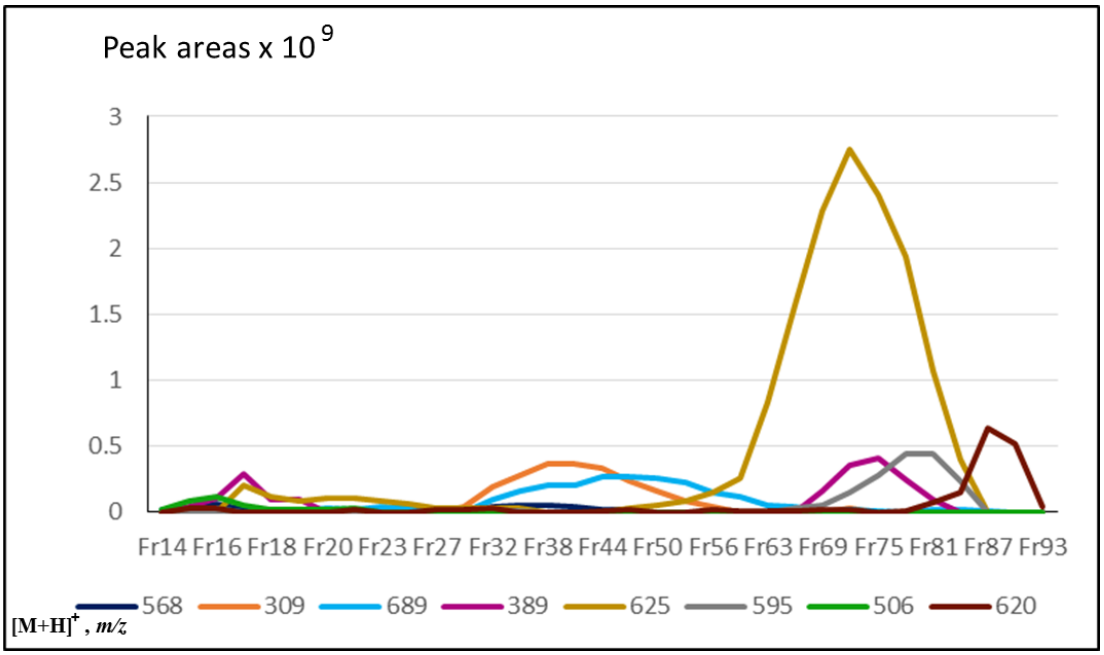


Figure 3-28: The distribution of non-pigmented compounds eluting from CPC separation based on the peak areas elucidated from LC-ESI-MS analyses

Based on the peak areas, the K_D values of all target compounds were calculated and are displayed in Table 3-13.

Table 3-13: K_D values of target compounds eluting from CPC separation of C18 enriched *O. dillenii* extract

m/z	551	637	568	309	689	389	625	595	620	727	549	358
K_D	0.17-0.41	0.34-0.82	0.72-1.24	0.72-1.34	0.72-1.66	2.01-2.32	1.80-2.42	2.11-2.53	2.53-2.74	2.01-2.32	0.17-0.31	0.13-0.48

The K_D values in this CPC approach exhibited distinct trends, in which the polar pigments showed lower values than that of HPCCC, while the less polar compounds showed converse results. For instances, the betanidin and feruloyl-betanin had higher K_D values compared to that in HPCCC run (2.01-2.32 vs. 1.25-1.61 and 2.01-2.32 vs. 1.25-1.56, respectively); whereas that of betanin was found to be lower (0.17-0.41 vs. 0.54-0.64). The resolution of several selected compounds were also calculated and displayed in Table 3-14.

Table 3-14: Calculated resolution of several pairs of targets compounds eluted by the CPC approach

R	$R_{1,2}$	$R_{2,3}$	$R_{3,4}$	$R_{4,5}$	$R_{5,6}$	$R_{6,7}$	$R_{7,8}$	$R_{1,4}$	$R_{1,6}$	$R_{1,3}$	$R_{1,12}$	$R_{1,14}$
	0.74	0.74	0.09	0.26	2.07	0,0	0.35	1.60	5.68	1.67	6.00	0.05

The $R_{i,j}$ values of this CPC run were very different from that of HPCCC, indicating variable fractionation efficiencies. The separation power in CPC was doubled for betanin and phyllocactin ($R_{1,2}$, 0.74 on CPC vs. 0.33 on HPCCC) and also higher than that of heated experiment (0.67). Likewise, better resolution was achieved in the pairs of phyllocactin and hydroxy-neobetanin ($R_{2,3}$, 0.74 on CPC vs. 0.25 on HPCCC), betanin and hydroxy-neobetanin ($R_{1,3,}$ 1.67 vs. 1.3) and so on. The highest resolution power was found within betanin and feruloyl-betanin, in which $R_{i,j}$ value was nearly two times higher than that of HPCCC ($R_{1,12}$, 6.0 vs. 3.2). Surprisingly, betanin and indicaxanthin were resolved by HPCCC with the resolution of 1.60 whereas that of CPC was much higher at 5.68. This result together with highly increased elution volume of indicaxanthin under CPC conditions can be explained by the strong effect of TFA. On the other hand, there was intensive co-elution of betaxanthin and hydroxy-neobetanin although they were partly fractionated in HPCCC experiments; the same situation was seen between betanidin and isorhamnetin rutinoside. Noticeably, the complete co-elution of the two flavanoid glycosides (**7**,**8**) were found different from the previous HPCCC runs, where they were effectively separated ($R_{7,8}$: 1.35). Only the compound **10**, exceptionally, was well separated from other unknown components; however, its low concentration made further identification impossible. These different $R_{i,j}$ values between CPC and CCC apparatus might relate to their typical phases mixing mechanism, in which CCC produces gentle wave mixing while CPC

generates more vigorous cascade mixing.[174] That requires more research efforts to confirm the differences between HPCCC vs. CPC operation, and a strategy should be developed for a better fractionation on CPC.

In general, the CPC fractionation of *O. dillenii* enriched extract resulted in the partial separation of betanin from its positional isomers gomphrenin I although the separation of the 15*S*/15*R* epimers was not successful. This CPC apparatus partly fractionated betanin from their degraded products, resulting in few fractions that seem to contain pure betanin. Nevertheless, most of the non-identified compounds were not separated effectively compared to the HPCCC approach. Since this was the first experiment using this solvent system on a CPC to purify betalains, these results proposed more research efforts to search for the optimal operating parameters such as speed, flow rate, temperature, etc. Additionally, the combination of CPC with an off-line LC-MS/MS analysis was able to monitor the fractionation of betanin stereoisomers, which were not possible from ESI-MS profiles method. However, the LC-MS measurements done for all the fractions eluting from a CCC/CPC processes require long experimental time and increase the chance of contamination within MS-interface and high-vacuum compartment.[163]

3.3.3 Conclusions

The standard 'betalains' solvent system TBMe/n-BuOH/ACN/water TFA was applied to both HPCCC and CPC systems for purification of *O. dillenii* C18 pigment extract resulting in a good fractionation of pigments from non-pigment compounds, particularly the significant resolutions of the betanin from betanidin and the non-polar compounds, such as flavonoids glycosides. However, the separation of other pigment derivatives were not effective, in which the dominant betanin was poorly resolved from its degradation products. These low resolutions indicated the insufficient ion-pair effects of TFA, and a stronger perfluoro carboxylic acid such as HFBA would be required for such polar mixtures. Because of the strong emulsification behavior of the extract, the carry-over of stationary phase was seen within all runs which could interfere with the fractionation mechanism. Nevertheless, the stationary phase leaking happens only in several initial fractions and was not taken into account in K_D calculation.

Historically, UV-Vis, HPLC and LC-MS have been widely used as on-line/off-line spectrometric methods to monitor the CCC separation. The hyphenation with LC-MS has become the technique of choice because of their sensitivity and high accuracy. Recently, there are many applications of a fast detection method in which the eluted fractions were sequentially injected into a high sensitivity and selectivity ESI-MS/MS to generate the full molecular weight profile of all ionizable compounds present in these fractions. The combination could visualize the distribution of selected ion traces among these injected fractions, enabling a fast and accurate

detection for target metabolites. Michel et al. (2013) summarized the recent progress in hyphenation of different CCC/CPC approaches with multiple detection methods for a better separation power and peak quality together with some successful applications on natural product purification.[175] For instance, this 'mass' target-guided screening method was applied successfully in monitoring and acquisition of structural information of betacyanin derivatives such as the acyl-oligosaccharide linked betacyanins from the *Bougainvillea glabra*, or that of apolar bioactive metabolites from Saudi-Arabian propolis and flavonoids from the *Impatiens glandulifera Royle flowers*, etc.[80,176,177] Nevertheless, this method is not able to distinguish the fractionation of the stereoisomer/epimers (such as betanin and isobetanin), and requires a second chromatography dimension such as C18 LC-ESI-MS.

3.4 THE PURIFICATION OF BETALAINS FROM THE COMPLETE PIGMENTED VIETNAMESE RED DRAGON FRUIT (*HYLOCEREUS POLYRHIZUS* VARIETY) BY HPCCC

3.4.1 Aim of this study

Although Vietnam is one of the top *Hylocereus spp.* exporters, there are few studies on the phytochemistry of the Vietnamese red dragon fruits which are local hybrids. This cultivar has higher nutritional values than the white flesh variety, particularly the betalain content within pigmented flesh and skins are characteristic. Wybraniec and Mizrahi (2002) proved that the betalain levels and its constitutents varied amongst different *Hylocereus* species; extraction methods and processing conditions also affected the pigment yields since these treatments could alter degradation and result in reduction of total pigment contents.[54,125,178] This study aimed to isolate the pigments of Vietnamese red dragon fruits by applying the chitin pre-purification method (cf. section 3.1.2.2) followed by the HPCCC separation, and to concentrate the minor betacyanin pigments which were not detectable by the ESI-LC-MS evaluation of the crude extract.

The application of CCC for fractionation of *Hylocereus polyrhizus* pigments was done before using multiple ion-pair assisted solvent systems (cf. Table 2.1); in which both perfluoro carboxylic acids such as trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA) were used on a HSCCC device (Pharma Tech, model 1000). That study included systems modified with 0.7 – 1% HFBA and could partly separate phyllocactin, hylocerenin and betanins while their artifacts were not sufficiently resolved.[73] Despite of the strong ion-pair interactions with highly polar betalains, the persistent fluorinated reagents such as HFBA are not encouraged in term of food grade chemicals since their occurences in the fractions afterwards are problematic

for bioassays. Additionally, this strong acid is harmful for betalains by inducing a certain degree of decomposition, its high amphiphilic character also causes emulsification during CCC operation by hampering system equilibrium or retention of stationary phase. In order to reduce these risks of using HFBA, this study used the less drastic ion-pair TFA with the typical solvent system of TBMe/n-BuOH/ACN/water (v/v/v/v, 2/2/1/5, 1% TFA) applied on a robust HPCCC. The collected fractions were evaluated by LC-ESI-MS/MS; and the whole procedure used in this study is summarized in Figure 3-29.

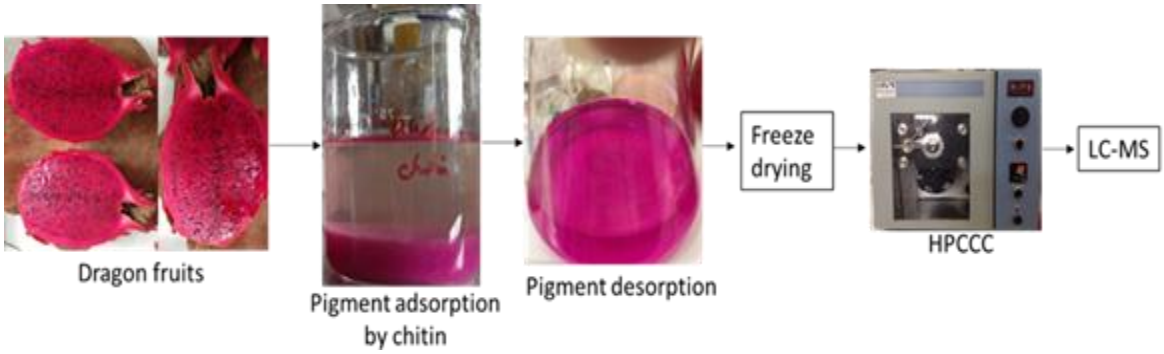


Figure 3-29: Summarized procedure to study the betalains from Vietnamese red dragon fruits using ion-pair HPCCC with off-line LC-ESI-MS/MS analysis

3.4.2 Results and discussion

3.4.2.1 LC-MS/MS evaluation of crude extract and chitin enriched extract

The LC-ESI-MS/MS screening of crude extract for identification of target compounds was described in previous experiment (section 3.1.2.2a), where several betacyanins were found such as 15*S*/15*R* betanin, 15*S*/15*R* phyllocactin, 15*S*/15*R* apiosyl-betanin, 15*S*/15*R* betanidin-5-O-*β*-sophoroside and 15*S*/15*R* hylocerenin as C15 epimeric mixtures. The dominant betaxanthin, indicaxanthin, was also recognized similarly to previous studies in this red flesh cultivar. The higher sugar and pectin contents compared to the pigments concentration caused difficulties for the HPCCC experiments, which needed an enrichment process to remove most of these interfering compounds for pigments concentration. After pre-purification by the chitin process (cf. section 3.1.2.2), the enriched extract was examined by LC-MS to overview the pigments profile, and the resulting chromatogram is showed in Figure 3-30. In comparison to the crude extract, many unknown signals representing the complex fruit metabolites such as organic acids and reducing sugars disappeared, while the pigments signals were significantly enhanced. This confirmed the excellent betalain adsorption capacity of chitin material.

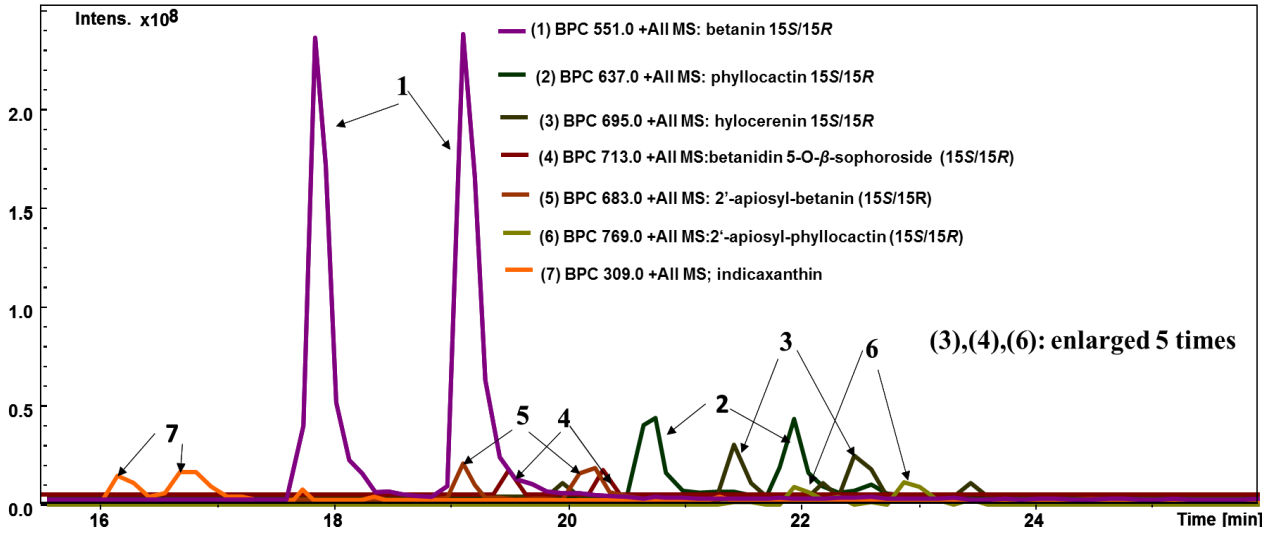


Figure 3-30: ESI- LC-MS chromatogram of the pigment extract enriched by chitin adsorption

As expected from the crude extract, betanins (**1**), phyllocactin (**2**) and 2'-apiosyl-betanin (**5**) were the dominant betacyanins next to several minor concentrated pigments such as hylocerenin (**3**), 2'-apiosyl-phyllocactin (**6**) and trace of betanidin 5-O-*β*-sophoroside (**4**); these minor structures were clearly detected within this enriched sample although they were found in trace amounts in the crude extract. The signals of degraded betanins namely mono decarboxy-betanin and neobetanin were also recognized in significant amount (data not shown). The increases in the ratio of 15*R* to 15*S* stereoisomers of both betanin and phyllocactin were noticed similarly to the study of Iatteui and Minale (1964), from which isomerization of betanin and phyllocactin was enhanced under low pH, causing the enhancement of the 15*R* epimer ion intensity compared to it lower level occurred in the crude extract (cf. Figure 3-2).[32] The induced isomerization within chitin process was noticed in the previous experiments, which might be due to the activation of chitin material by acidic pH and pigments desorption by ammonium acetate. Interestingly, there were some minor pigments identified in the *Hylocereus spp*. previously such as 5"-O-E-sinapoyl-2'-apiosyl-betanin and 5"-O-E-sinapoyl-2'-apiosyl-isobetanin;[126] however, these compounds were not found in this Vietnamese cultivar. This result showed the differences in pigment production/ expression depending on genotypes and was in agreement with the study of Wybraniec and Mizrahi (2002).[54] The main pigments detected from this chitin enriched extract are summarized in Table 3-15.

Table 3-15: The main pigments found in Vietnamese red dragon fruit enriched extract

Number	Name	λmax (nm)	*m/z* $[M+H]^+$	MS^2 *m/z*
1	Betanin (15*S*/15*R*)	538	551	389 (100)
2	Phyllocactin (15*S*/15*R*)	536	637	551 (29.26), 389 (100)

3	Hylocerenin (15*S*/15*R*)	539	695	551 (100), 389 (9.46)
4	Betanidin 5-O-*β*-sophoroside (15*S*/15*R*)	539	713	551(8.87), 389 (81.69)
5	2'-apiosyl-betanin (15*S*/15*R*)	538	683	551 (29.26), 389 (100)
6	2'-apiosyl-phyllocactin (15*S*/15*R*)	541	769	683 (18.5), 389(100)
7	Indicaxanthin	483	309	265(100)

3.4.2.2 Ion-pair (IP) HPCCC separation with LC-ESI-MS/MS analysis of betalains from Vietnamese dragon fruits (Hylocereus polyrhizus)

The replacement of preparative HSCCC (spinning velocity 1000 rpm, centrifugal force field of 60 x g) by the robust HPCCC (spinning velocity 1600 rpm, centrifugal force field of 240 x g) allowed the increase of flow rate (4.0 ml/min vs. 3.0 ml/min) and a better retention of stationary phase (76.8% vs. 56%) using the standard solvent system of TBMe/n-BuOH/ACN/water 1% TFA for betalains.[76] This HPCCC separation operated at highest speed of 1600 rpm was used to fractionate approximately 350 mg pigment enriched extract by chitin adsorption. After the breakthrough of mobile phase at fraction 20; all pigmented fractions collected from HPCCC run were analyzed by LC-ESI-MS/MS analysis.

The pigments rapidly eluted within the short range of fractions from 21 to 36, where most of the dominant compounds were found co-eluting. Based on the peak areas of target compounds elucidated from LC-ESI-MS/MS chromatogram, the eluting profile of injected fractions is illustrated in Figure 3-31.

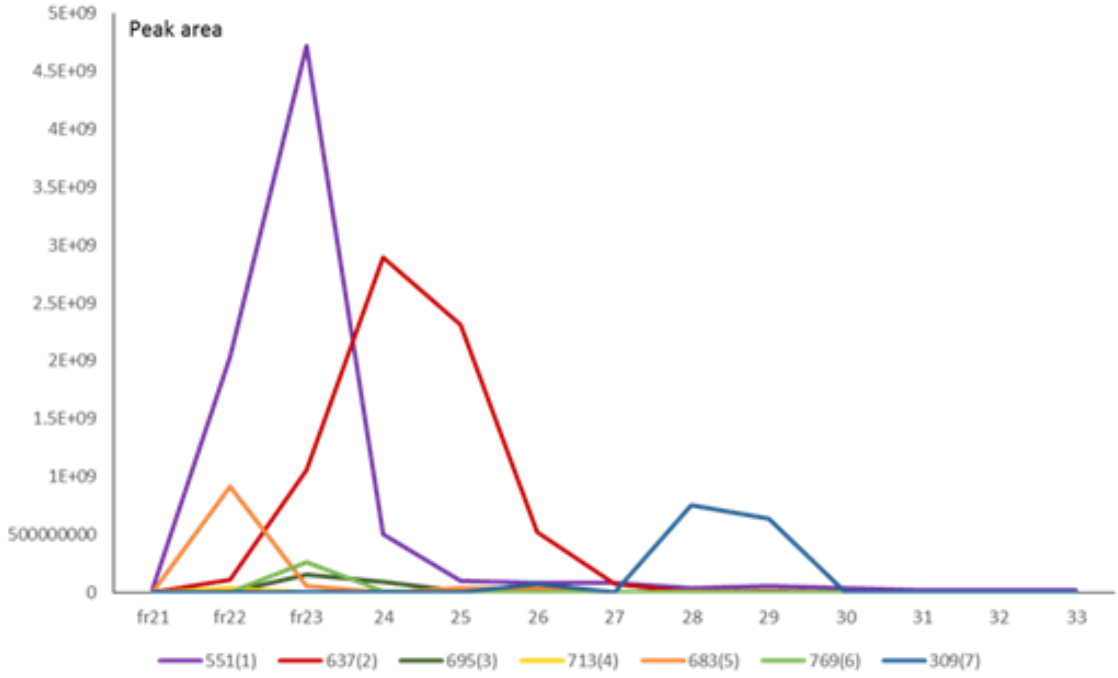
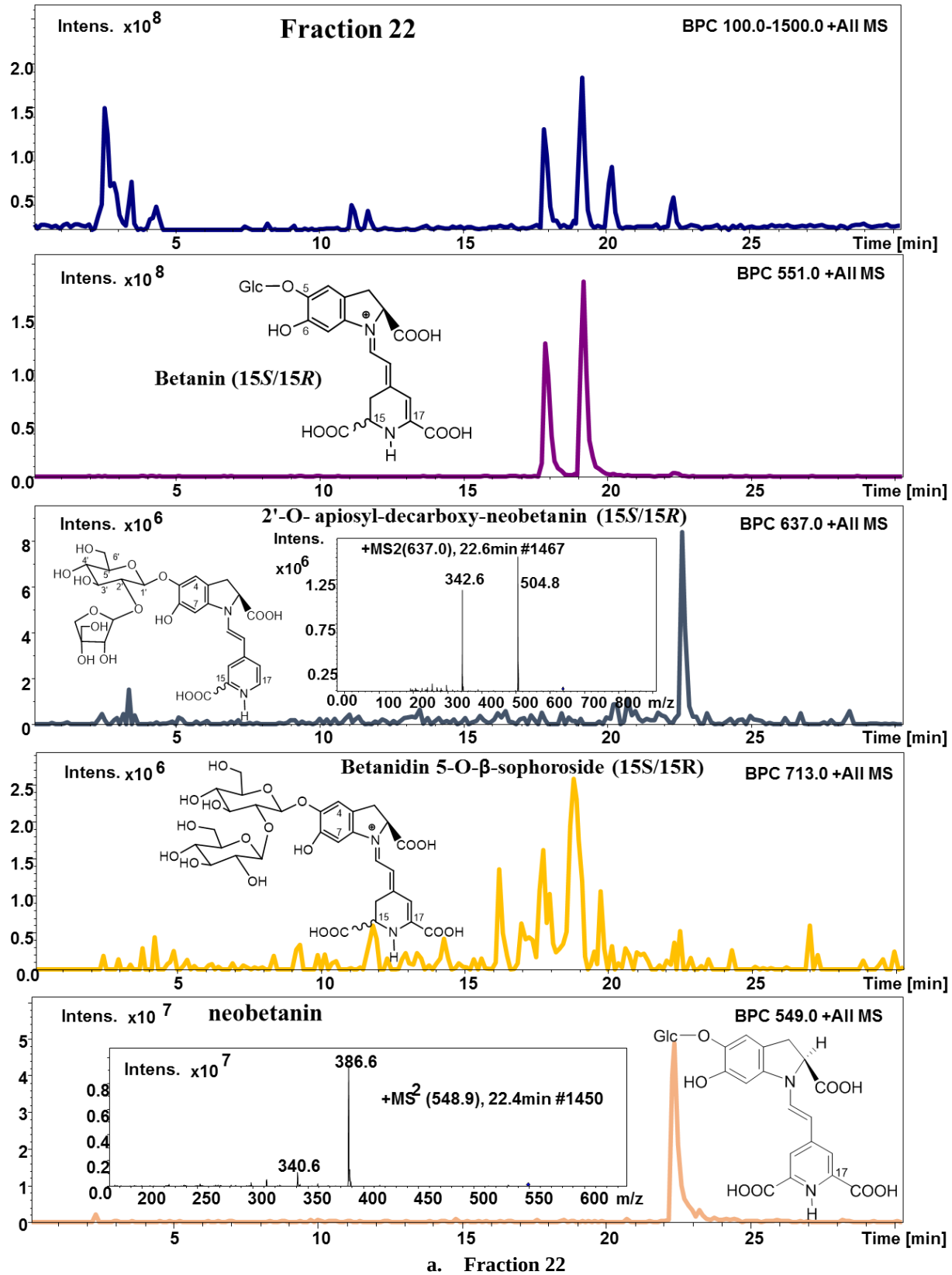


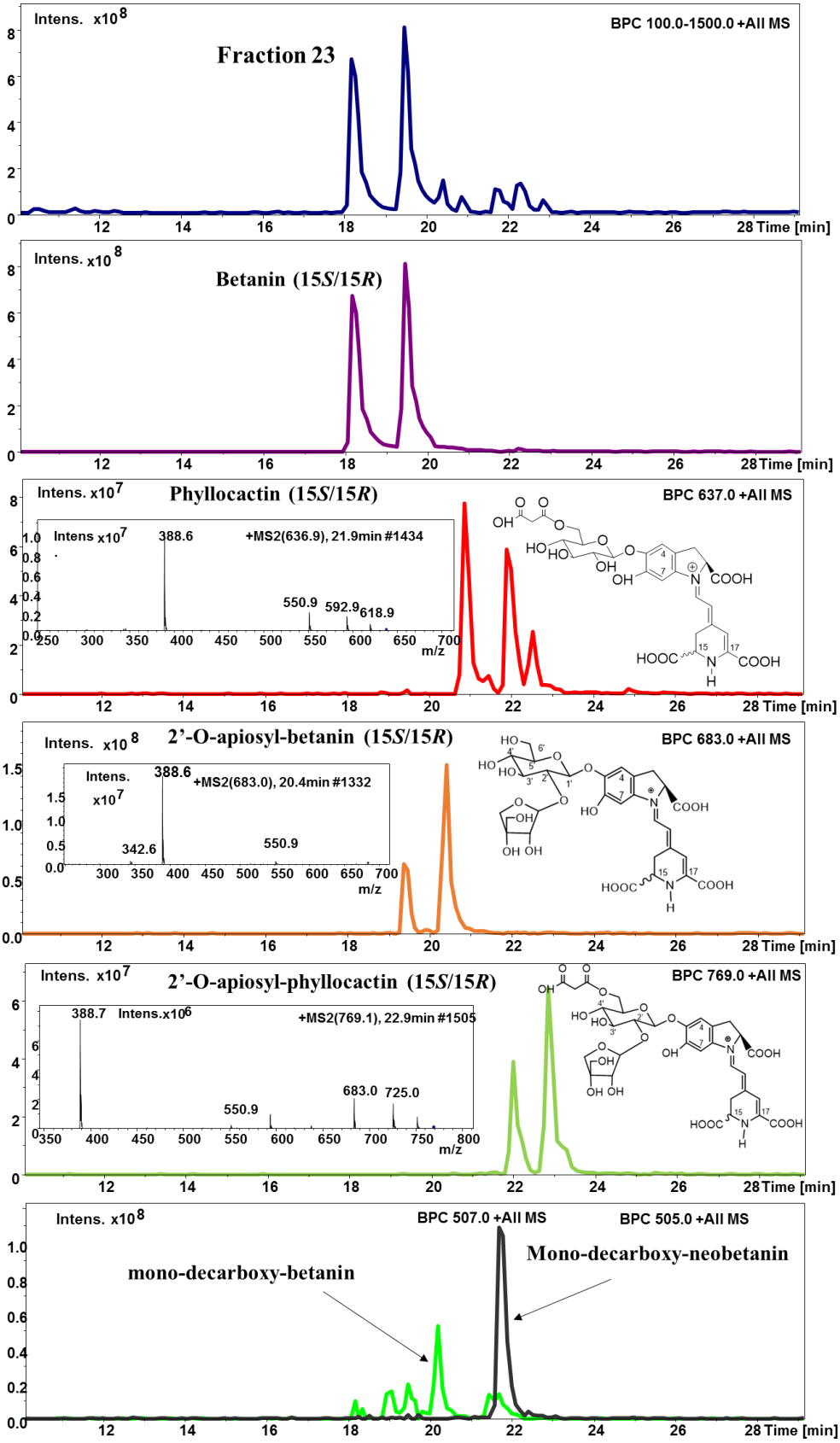
Figure 3-31: The eluting profile of target pigments elucidated from LC-ESI/MS chromatogram of collected fractions

As can be seen, the most dominant pigments, betanin/isobetanin (**1**), started to elute early and highly concentrated in fraction 23, followed by phyllocactin/isophyllocatin (**2**) occurring significantly in fraction 24 while 2'-apiosyl-betanin (15*S*/15*R*, **5**) appeared mostly in fraction 22. These results showed to be identical with the separation of Wybraniec et al. (2009)[73] where **5** was found only in traces and co-eluted with betanins within very initial fractions. 15*S*/15*R* betanidin 5-O-*β*-sophoroside **(4)** was also detected in the fraction 22. Noticeably, there was ion signal $[M+H]^+$ at *m/z* 637 which was identical to that of phyllocactin within the fraction 22; however, the fragmentations were different at *m/z* 345 and 505 with neutral loss of Δ*m/z* 132 and 162 indicating the loss of apiosyl and glucose units. This signal could represent for decarboxy-neo-2'-apiosyl-betanin. The overlapping of these highly polar structures **4**, **5** found similarly to the previous HPCCC separation, in which the high polarities give them a strong affinity to the mobile phase and small elution volume at the beginning of the collected sequence. 15*S*/15*R* hylocerenin (**3**) and 15*S*/15*R* 2'-apiosyl-phyllocactin (**6**) were noticed mostly in fraction 23 and were not fractionated from phyllocactins and betanins. Despite of the insufficient fractionation capacity of dominant pigments, the minor ones seem tobe fractionated better from each other. For instance, hylocerenin (**3**) was partly apart from the pigment **5** and **4** similarly to **6**. Noteworthy, the elution orders of hylocerenin and 2'-apiosyl-betanin was opposite to that from the study of Wybraniec et al. (2009)[73] which might be due to their reverse concentration within two varieties. The ESI-LC-MS chromatogram of several pigmented fractions with selected ion traces of interest are displayed in Figure 3-32 (a-d).

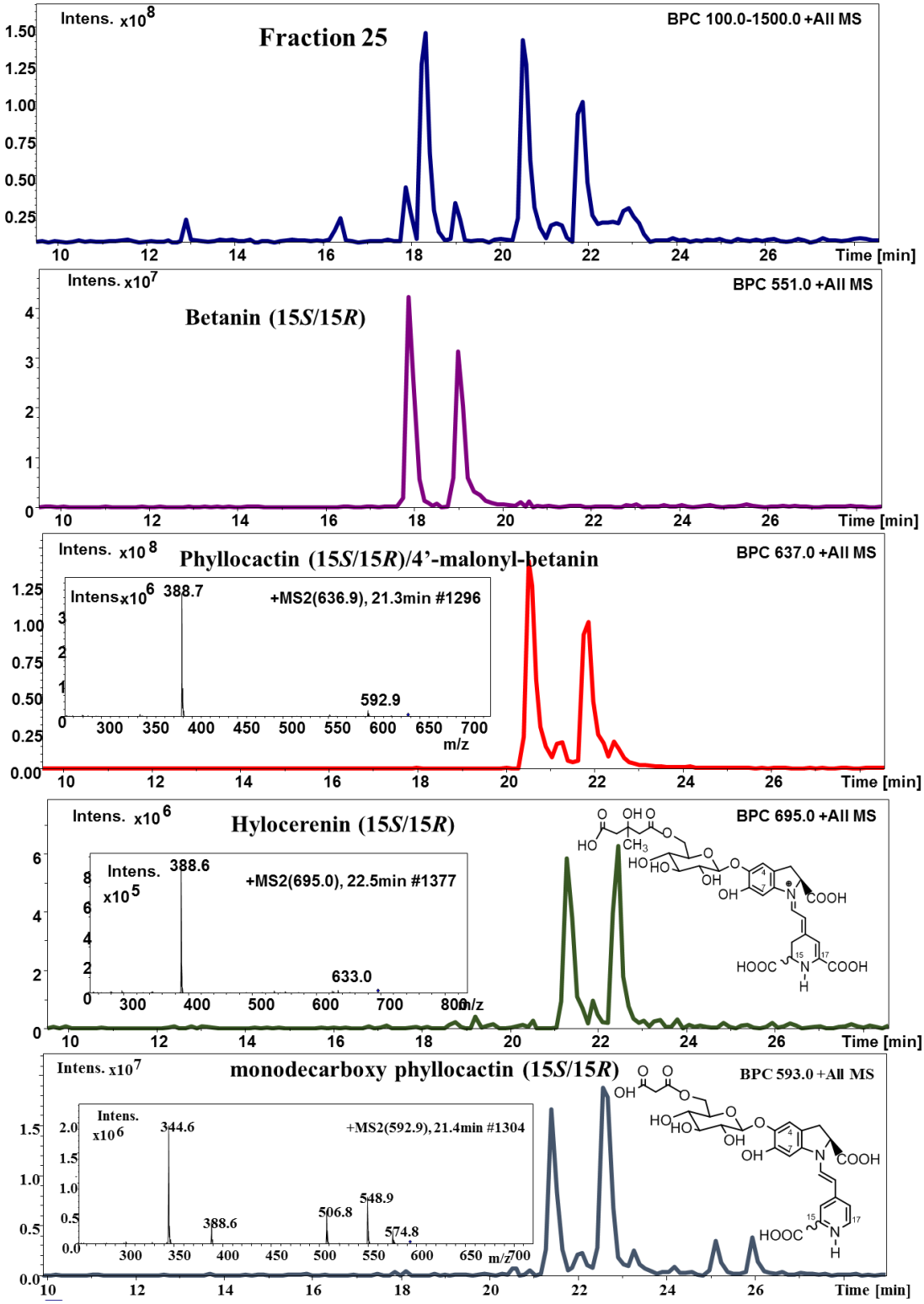


a. Fraction 22

Figure 3-32: LC-ESI-MS/MS chromatograms with selected ion traces of few pigmented fractions

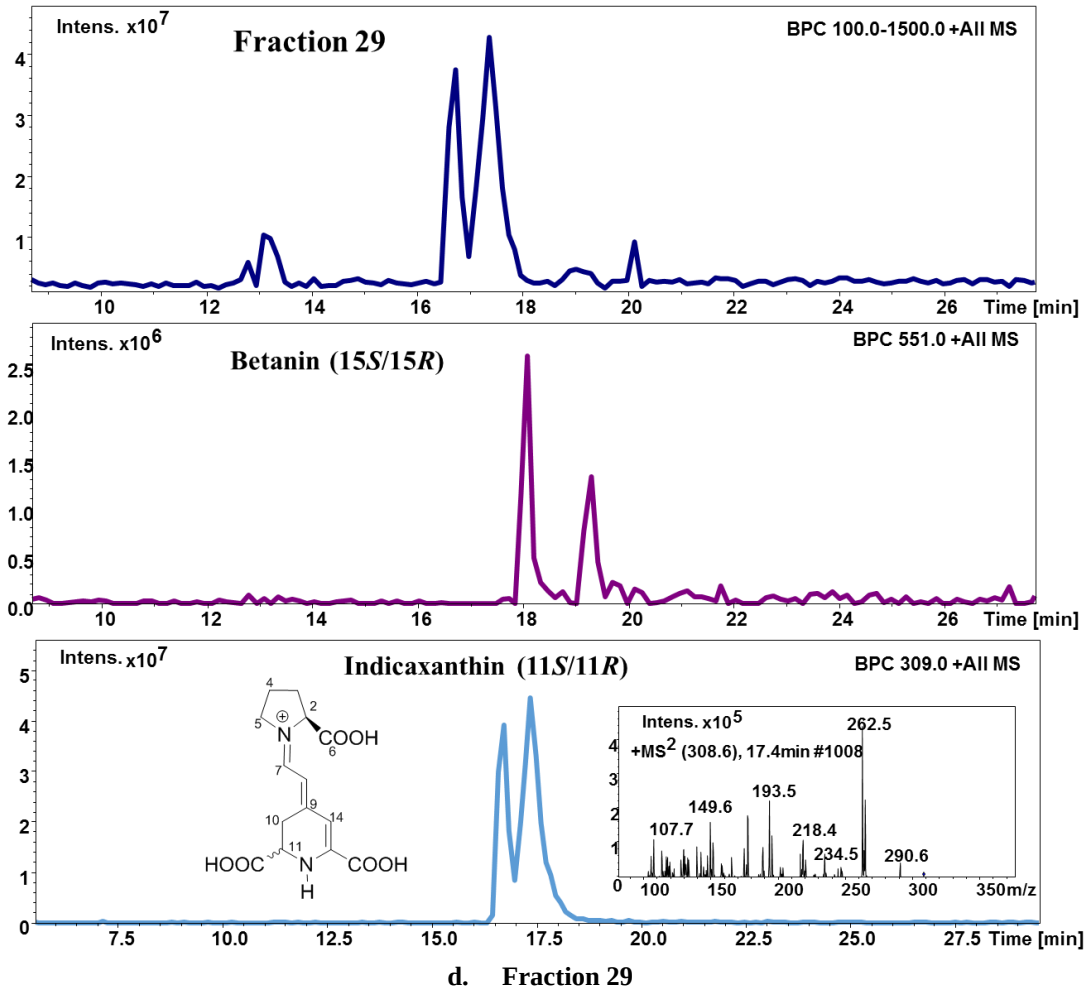


b. Fraction 23



c. Fraction 25

Figure 3-32 (cont): LC-ESI-MS/MS chromatograms of few pigmented fractions



d. Fraction 29

Figure 3-32: LC-ESI-MS/MS chromatograms of few pigmented fractions

There were clearly two extra peaks displaying $[M+H]^+$ at *m/z* 637 next to phyllocactin/isophyllocactin (Figure 3-32c, Fraction 25); their MS/MS characteristic fragments at *m/z* 551, 389 through two neutral losses of 86 could indicate the epimers 4'-malonyl-betanins (15*S*/15*R*). Wybraniec (2008) suggested that these isomers were formed by the acyl migration between two adjacent hydroxyl groups at C-4' and C-5' of the glucose units within phyllocactins/isophylocactin structures.[179] On the other hands, only two signals exhibited at *m/z* 713 with fragmentations to *m/z* 551, 389 and cleavages of two hexoside units corresponding to betanidin 5-O-*β*-sophoroside (15*S*/15*R*); whereas up to six similar peaks (un-identified) were found in the previous HSCCC experiments of Wybraniec et al. (2009). This could refer to the distinct pigment profiles of each *Hylocereus spp.* and was in agreement with the study of Wybraniec and Mizrahi (2002) on the phytochemistry of several red dragon fruit cultivars.[54]

As expected from the experiments of *O. var dillenii* above, this solvent system was able to separate indicaxanthin from most of the betacyanins due to its strong interaction with the ion-pair

agent TFA; that was also similar to the HSCCC separation of dragon fruit pigments before. Noticeably, the elution order of betaxanthin and several minor pigments such as 2'-apiosyl-betanin, hylocerenin and 2'-apiosyl-phyllocactin were not identical to their elution order on the LC-MS screening of crude extract (cf. Figure 3-30), which could be caused by the ion pairing effect of TFA. The high polarities of the functional groups in the pigments made it problematic for HPCCC fractionation compared with the fractionation of *Opuntia* pigments. The reversed elution orders between C18 RP-HPLC and HPCCC (*head to tail*) were noticed before, especially in the highly hydrophilic mixtures. That phenomena is believed to be due to the changed affinities to the stationary phases caused by formation of 'hydrophobic' cluster due to intermolecular associations amongst polar structures.[180]

In addition to the principle pigments, several signals detected at low ion abundance represented degraded pigments, namely 2-, 17- mono-decarboxy-betanin (*m/z* 507, MS^2 345), neobetanin (*m/z* 549, MS^2 387), neophyllocactin (*m/z* 681, MS^2 387) neo-decarboxy-betanin (*m/z* 505, MS^2 343), decarboxy-2'-apiosyl-betanin (*m/z* 639, MS^2 345) and cyclo-dopa-5-O-glucoside (*m/z* 358, MS^2 196), occuring in most of the pigmented fractions. The occurrences of these artifacts were enhanced during chitin pre-purification process similarly to the section 3.1, particularly in fraction 23. Whereas the 2- and/or 17-mono-decarboxy-phyllocactin (*m/z* 593) eluted slightly later in fraction 25 because of its higher lipophilic structures. The degradation of highly polar and labile betacyanins during ion-pair HPCCC separation was not only recognized in dragon fruit pigments but also for *Opuntia ficus indica* pigments described before.[73,45] Noticeably, the abnormal ratio of the epimers (such as 15*R*/15*S)* found in all pigments was also seen, particularly within the two principle betacyanins (**1**, **2**) and indicaxanthin (**7**). Surprisingly, the isobetanin (Fraction 23, Figure 3-32b) and iso-indicaxanthin (Fraction 29, Figure 3-32d) appeared in higher levels compared to their *S* counterparts. These superior levels of 15*R* isomers could result from the isomerization during chitin clean-up process using ammonium acetate.[32]

In comparison to the *Opuntia* experiment above, the increase of TFA concentration from 0.7% to 1.0% not only enhanced the distribution of pigments into the organic phase but also affected the retention value of stationary phase. For instance, 1% TFA effectively moved betalains to stationary phase and slightly reduced the retention of stationary phase compared to that of 0.7% TFA (76.8% vs. 80%, pigments started eluting from fraction 18 vs. fraction 20, respectively). This effect of TFA on stationary phase retention was in agreement with other studies on the evaluation of different solvent systems applied for betalains; from which TFA was found having emulsifying properties to specific solvent systems.[45,73]

3.4.2.3 Distribution values K_D and resolution factors of the HPCCC separation

From the LC-ESI-MS peak integration, the actual K_D values of the principle pigments were calculated based on the peak areas and results are displayed in Table 3-16. In general, the increase of TFA concentration promoted the ion-pair forming effect and resulted in slightly reduction of stationary phase retention and enhancement of pigment elution volumes due to more interaction to dissociated and charged molecules. In consequence, these effects caused higher K_D values than that of the *Opuntia* separation, particularly the value of betanin and phyllocactin (0.77 vs. 0.55 and 0.73 vs. 0.63, respectively), whereas the K_D of indicaxanthin was not very largely different (0.91 vs. 0.87). This result agreed with the prediction study of Wybraniec et al. (2009) that the higher TFA concentration could enhance the K_D values of betacyanins. Nevertheless, there were large offsets between the predicted K_D values by LC-ESI-MS analysis of phase layers and the actual values elucidated from this HPCCC study using elution volume of pigments (data not shown). The differences in sample cultivars and the pre-purification methods might strongly affect the equilibrium of the solvent systems within two HSCCC/HPCCC separations. In addition, the carry-over of stationary phase after sample injection was observed in all of the betalain separations (data not shown), that could be caused by significant emulsifying effect and hydrophilicity of betalains. These reasons in combination might cause the variation in K_D values between this HPCCC separation on dragon fruit pigments and previous HSCCC experiments on *Opuntia ficus indica* and *Hylocereus spp*.[45,73]

Table 3-16: Distribution factor K_D of target pigments elucidated from LC-ESI-MS data

Peaks	1	2	3	4	5	6	7
K_D	0.66-0.95	0.71-0.83	0.66-0.83	0.62-0.83	0.62-0.87	0.66-0.83	0.87-0.91

Similarly, the resolution factors $R_{i,j}$ were calculated for several pairs of pigments of interest (Table 3-17) in order to investigate the separation capacity of this HPCCC apparatus.

Table 3-17: Resolution factors of several pairs of target pigments separated by HPCCC

$R_{i,j}$	$R_{1,2}$	$R_{2,3}$	$R_{3,4}$	$R_{4,5}$	$R_{5,6}$	$R_{6,7}$	$R_{1,3}$	$R_{1,5}$	$R_{1,7}$
	0.13	0.22	0.50	0.00	0.22	1.67	0.00	0.19	0.71

In general, small $R_{i,j}$ values indicated a limited fractionation capacity of HPCCC, in which several pairs of pigments completely chromatographical co-eluted, such as betanin and hylocerenin ($R_{1,3}$), or 2'-apiosyl-betanin and betanidin 5-O-β-sophoroside ($R_{4,5}$). The co-elution

of betanin and phyllocactin ($R_{1,2}$) was expected similarly to the HSCCC separation; whereas the better resolution of phyllocactin and hylocerenin ($R_{2,3}$) was achieved on HPCCC despite the fact that they almost overlapped in HSCCC run using TFA.[73] Interestingly, the better resolution of these two compounds **2** and **3** was seen on this HPCCC using TFA although they were previously well fractionated on the HSCCC device using the strong ion-pair reagent HFBA (1%). The best fractionation was recognized for 2'-apiosyl-phyllocactin and indicaxanthin ($R_{6,7}$), followed by betanin and indicaxanthin ($R_{1,7}$). As the high velocity spinning HPCCC system using the same solvent TBMe/n-BuOH/ACN/water 1% TFA retained better the stationary phase on the coil-column system than the HSCCC device; this improved the result and better resolution of pigments separation might be explained.

3.4.3 Conclusions

This was the first time that enriched pigment extract from Vietnamese dragon fruit (*Hylocereus polyrhizus*) were separated by a HPCCC apparatus using TBMe/n-BuOH/ACN/water 1% TFA solvent system. The combination of the chitin pre-purification process and high performance CCC concentrated minor compounds into several fractions, allowed a better LC-ESI-MS/MS detection in the second chromatographic dimension compared to the direct crude extract analysis. It was able to separate betacyanins from indicaxanthin and some minor concentrated betacyanin structures such as 2'-apiosyl-phyllocactin. However, stronger ion-pair forming additives e.g HFBA would be required for a better resolution of such hydrophilic mixtures. Many attempts have been done to replace these drastic ion-pair acids and resulted in aqueous ammonium sulphate solvent system which are not compatible with MS studies.[82] In short, this solvent system with TFA was successful in fractionation of the polar pigments extract from *O. dillenii* but was not able to resolve the highly polar pigments from *Hylocereus polyrhizus* fruits.

3.5 THE STUDY OF THE RED PIGMENTED *ATRIPLEX HORTENSIS VAR RUBRA* METABOLITES BY ION-PAIR HPCCC

3.5.1 Aim of this study

Atriplex hortensis var rubra (*Atriplex*) is one of the oldest leafly vegetable displaying high content of bioactive secondary metabolites such as betalains, flavonoids glycosides and uncommon sulphated flavonoids. Although its metabolites and nutrition values have been occasionally revealed, a detailed report on *Atriplex* betacyanins was barely found. This study aimed to investigate the betalains and flavonoid glycosides from *Atriplex* leaves using the all-liquid HPCCC chromatographic approach. Due to the complex composition of the plants (cf. LC-ESI-MS/MS analysis of the crude extract, section 3.1.4.2d), a pre-purification step was

required prior to the HPCCC operation. The *Atriplex* crude extract was individually submitted to the enrichment processes by C18 resin and chitin in order to compare the pigment adsorption abilities of both materials. In another approach, a sephadex LH20 column was also used for flavonoid pre-purification before preparative HPCCC has been carried out. The collected fractions from pigment/flavonoid separations were evaluated by either ESI-MS/MS profile or TLC method, from which purified fractions were used directly for structural identification by LC-ESI-MS or 1D/2D-NMR. Figure 3-33 and Table 3-18 summarize the HPCCC experiments and the applied experimental parameters.

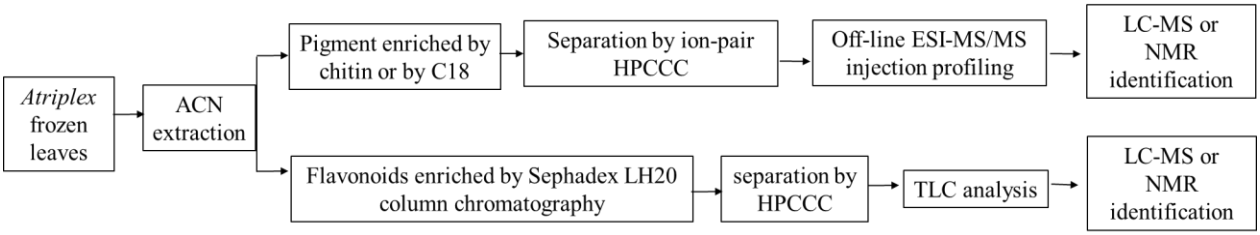


Figure 3-33: Workflow of the experimental procedure used within this study

Table 3-18: Summary of HPCCC separations done within this studies

Clean up method/ Material	HPCCC Solvent systems (v/v/v/v)	HPCCC injection m_{sample} (mg)	Flow rate	Stationary phase Retention
Chitin adsorbate	TBMe-n-BuOH-ACN-H_20-TFA (2/2/1/5/0.7%)	575	4.0ml/min	68.6%
C18 adsorbate	TBMe-n-BuOH-ACN-H_20-TFA 2/2/1/5/0.7%)	460	4.0ml/min	66.6%
Sephadex (polar part) fraction	n-Hexane-n-BuOH-H_20 (1/1/2)	97	4.0ml/min	70.0%
Sephadex (non-polar part) fraction	EtOAc-MeOH-H_20 (25/1/25)	75	4.0ml/min	69.0%

3.5.2 Results and discussion

3.5.2.1 LC-ESI-MS/MS evaluation of the crude extract

The ACN extract was screened by LC-MS/MS to identify the target compounds (results are summarized in the section 3.1.4.2d). Amaranthin (2"-O-E-feruloyl, **1**), celosianin II ((2"-O-E-

feruloyl)-amaranthine, **4**) and their C15 stereoisomers were the dominant pigments next to traces of betanin (**2**) and cellosianin I ((2"-O-E-4-coumaroyl)-amaranthine, **3**). Two unknown structures **5** and **6** eluted lately and appeared as the main flavonoid glycosides next to minor signals of sulphated flavonoid glycosides. Signals of degraded pigments such as dehydrogenated and decarboxy - derivatives were also detected through their characteristic fragment ion at *m/z* 387 and 345, respectively. Noticeably, plenty of non-identified peaks appeared and strongly overlaid the pigments of interest. Therefore, that complex extract required a pre-purification step to eliminate the interfering compounds and to concentrate the pigments before the HPCCC operation. The compounds of interest are summarized in Table 3-19 below.

Table 3-19: Target compounds from *Atriplex hortensis varubra* analysis by LC-ESI-MS of crude extract

Peak N°	**Name**	***m/z* $(M+H)^+$**
1	amaranthin (2"-O-E-feruloyl)	727
2	betanin	551
3	celosianin I ((2"-O-E-4-coumaroyl)-amaranthine)	873
4	celosianin II ((2"-O-E-feruloyl)-amaranthine	903
5	quercetin-O-malonyl glucoside	551
6	kaempferol-O-malonyl glucoside	535
9	kaempferol-3-O-sulphate-7- O α- arabinopyranoside	499
8	kaempferol-O-pentoside	419

3.5.2.2 The purification of Atriplex pigments by HPCCC monitored by ESI-MS/MS profile – Comparison of betalains adsorption abilities of chitin and C18 resin

a) HPCCC separation of the pigment enriched by chitin – ESI-MS/MS injection profile of eluted HPCCC fractions

In this experiment, pigments pre-purified by chitin (so called chitin adsorbate) was fractionated in the first step on a HPCCC spectrum using the ion-pair solvent system TBMe/n-BuOH/ACN/water/TFA and the collected fractions were monitored by ESI-MS injection approach. The molecular weight profiling of target metabolites is displayed in Figure 3-34 where eluates started eluting after the breakthrough of mobile phase up to fraction 21.

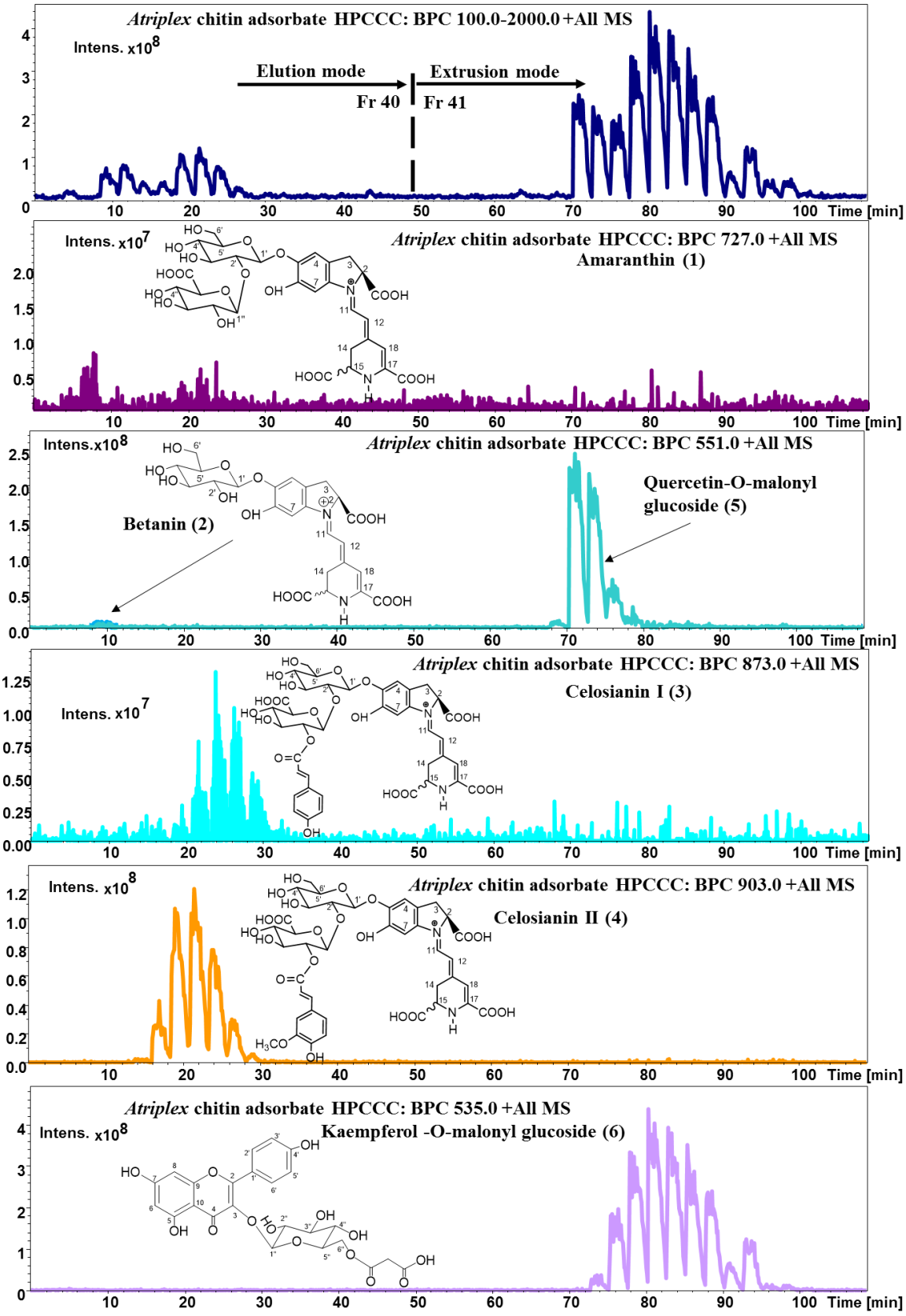


Figure 3-34: ESI-MS/MS injection profile and selected ion traces of fractions eluted from HPCCC separation (*head to tail*) of *Atriplex* pigments adsorbed by chitin

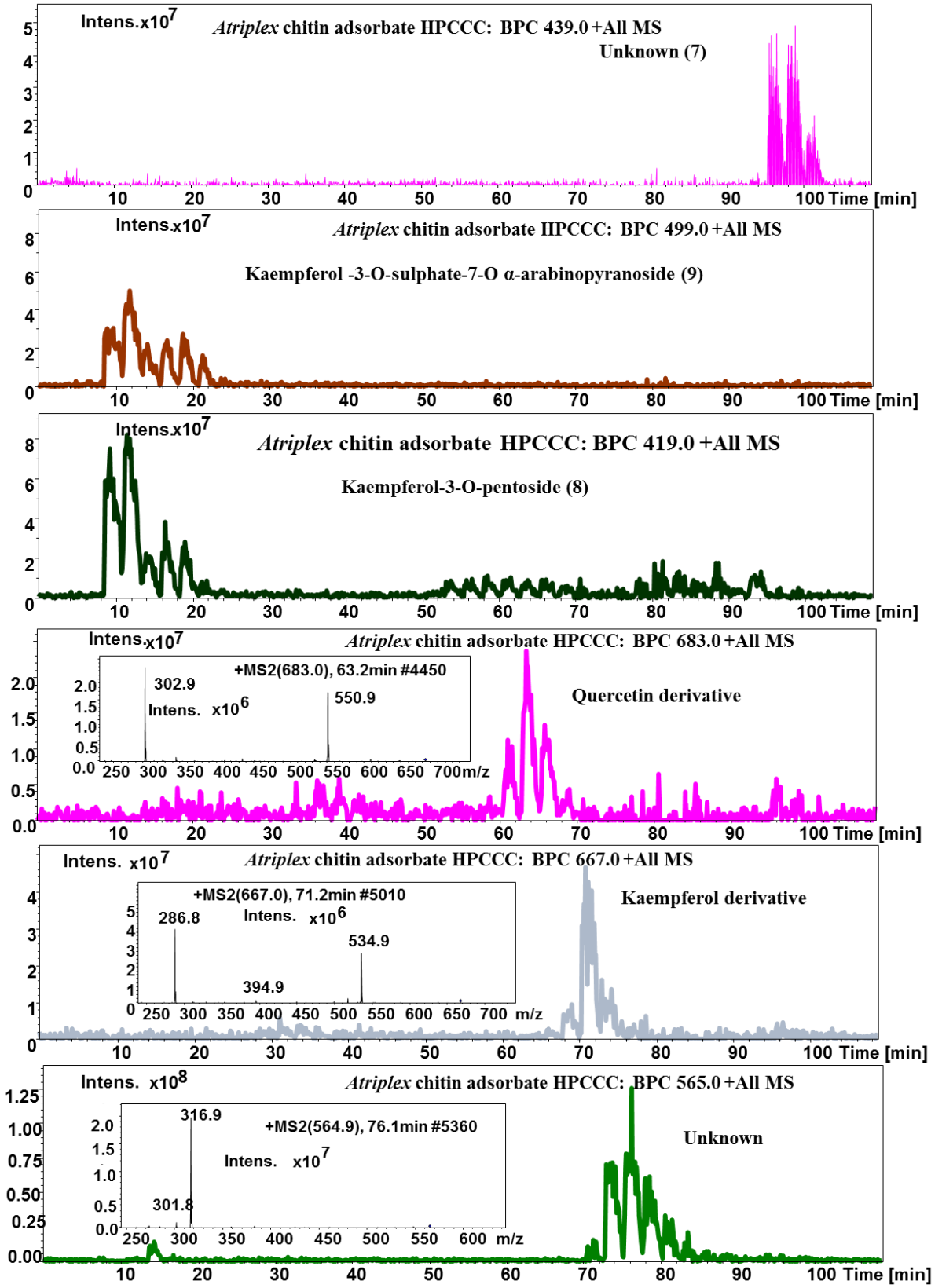


Figure 3-34 (cont): ESI-MS/MS injection profile and selected ion traces of fractions eluted from HPCCC separation (*head to tail*) of *Atriplex* pigments adsorbed by chitin

This approach was able to fractionate approximately 500 mg of pre-purified chitin adsorbate, resulting in a good separation of the pigments and non-pigmented compounds. Similarly to the C18 RP LC-MS elution pattern observed for the crude extract, celosianin II was found as the principle pigment exhibiting at *m/z* 903 (positive mode) next to traces of amaranthin (*m/z* 727), celosianin I (*m/z* 873) and betanin (*m/z* 551); these betalains were recognized by their typical daughter fragments at *m/z* 551 and *m/z* 389 representing for betacyanins and the aglycone betanidin. Amaranthin and betanin were detected at early elution volumes in HPCCC-ESI-MS profile (fraction 23 and fraction 24, respectively), which were identical to their short elution time in the LC-ESI-MS analysis. Interpretation of the selected ion traces in the HPCCC profile revealed that celosianin II (**4**) was well separated from amaranthin (**1**), betanin (**2**), and partly resolved from celosianin I (**3**). Surprisingly, the elution orders of **4** and **3** (within fraction 27 – 31 and fraction 29 – 32, respectively) were reversed to their observed elution orders from LC-ESI-MS where **4** had longer retention time due to its lower polarity. Despite of the co-elution, some fractions of **4** displayed high purity which were then used directly for 1D/2D-NMR experiments and structural identification to elucidate celosianin II (cf. section 3.5.2.4a).

Besides the betacyanin pigments, in these early eluting fractions the kaempferol derivatives exhibiting $[M+H]^+$ signal at *m/z* 499 (kaempferol 3-O-sulphate-7- O α- arabinopyranoside, **9**) and 419 (kaempferol-O-pentoside, **8**) were detected and characterized by the typical MS/MS fragment ion at *m/z* 287. These compounds were well separated from amaranthin, celosianin I and partly mixed with celosianin II. Meanwhile, other non-pigmented compounds were concentrated within later eluting fractions during the extrusion-mode. There were separations of quercetin-O-malonyl-glucoside (**5**), kaempferol-O-malonyl glucoside (**6**) and the unknown structure **7** (*m/z* 551, 535 and 439, respectively) in which the late elution of **7** was diametral to its prompt appearance in the LC-ESI-MS. Noticeably, the quercetin-O-malonyl glucoside (**5)** showed $[M+H]^+$ at *m/z* 551 which was similar to betanin (**2**); however, **5** fragmented differently at m/z 303 which indicated the quercetin derivative (Figure 3-34). As it can be seen, parts of **6** and **7** showed good purities, but their actual amounts were insufficient for further structural identification. Likewise, traces of minor flavonoid glycosides displaying molecular ion signals at *m/z* 683 (MS^2 551, 303) and *m/z* 667 (MS^2 535, 287) were detected in several early HPCCC fractions of extrusion-mode and were well separated from each other. The *m/z* 667 trace was found to co-elute with other unidentified signals at *m/z* 565, 435, etc. These minor signals were completely mixed with **5** but partly separated from **6**. These unknown structures were supposed to be flavonoids and will be discussed separately in the following section of flavonoid glycoside purification.

b) HPCCC separation of C18 adsorbate – ESI-MS/MS injection profile of eluted fractions

The ESI-MS injection profile and selected ion traces are shown in Figure 3-35.

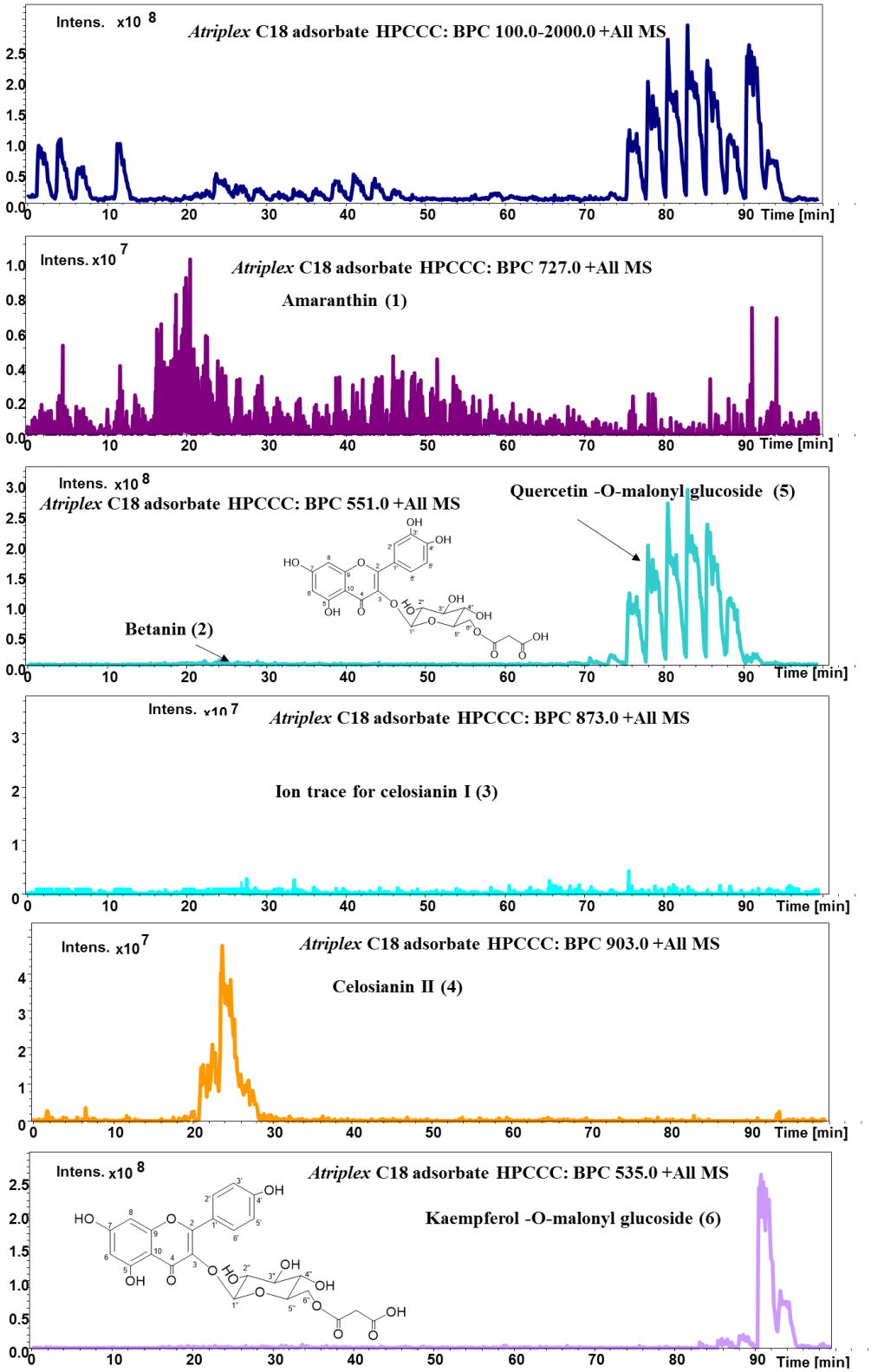


Figure 3-35: ESI-MS/MS injection profile and selected ion traces of fractions eluted from HPCCC separation of *Atriplex* pigments (*head to tail*) adsorbed by C18

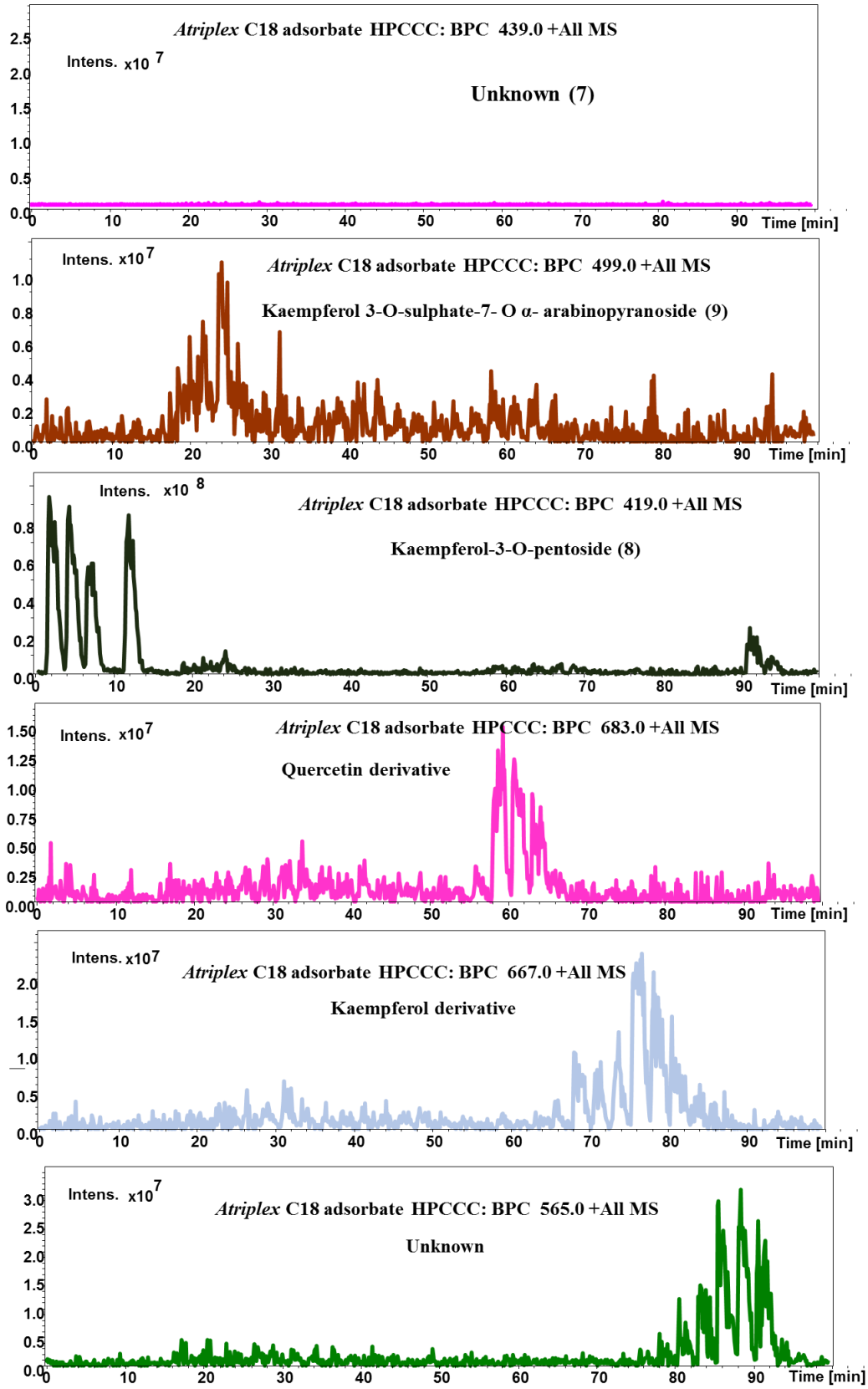


Figure 3-35 (cont): ESI-MS/MS injection profile and selected ion traces of fractions eluted from HPCCC separation of *Atriplex* pigments (*head to tail*) adsorbed by C18

The HPCCC separation of the C18 enriched adsorbate was done similarly to the preparative experiment on the chitin adsorbate, in which the components started eluting from fraction 15 after the breakthrough of mobile phase. As expected, this solvent system also resolved the pigment amaranthin from celosianin II and concentrated them into specific fraction ranges from 26 - 28 and 28 - 30, respectively. However, the mass profile of the C18 pigment adsorbate displayed several differences in comparison to that of the chitin clean-up, particularly the insignificant signal intensites of pigments betanin and celosianin I. The disappearance of these two pigments indicated the composition in the metabolites of these two pre-purified extracts, which were caused by the selective adsorption capabilities of betalains to C18- and chitin-materials.

Interestingly, the unknown compound **7** was not detected in any of the HPCCC fractions while traces of flavonoid glycosides quercetin and kaempferol derivatives (*m/z* 683 and 667, respectively) were well separated similarily to the previous run. The elution orders of these compounds were identical between the two HPCCC runs except the distribution of the two compounds quercetin-O-malonyl glucoside (**5**) and kaempferol-O-malonyl glucoside (**6**). **6** was highly concentrated in earlier eluting fractions than **5** whereas that was observed to be reversed in chitin adsorbate. Additionally, the above experiment of chitin adsorbate could resolve **5** and **6** from the unknown traces of *m/z* at 565 and 435, whereas in this separation this was not achieved. These unknown structures eluted very late within the elution-mode but their actual amounts were not sufficient for a further structural investigation. Surprisingly, traces of unknown pigments displaying at *m/z* 889 (MS^2 at 551 and 389) and degraded betalains were not recognized in both HPCCC separations indicating a gentle processing, whereas they were recognized in the HSCCC operation described before.[85]

c) The comparison of the partition coefficient K_D values and resolution factor values $R_{i,j}$ of *Atriplex* – HPCCC separations (chitin and C18 clean up)

In order to study the effects of the samples composition on the HPCCC separation, the specific K_D values of several compounds of interest were calculated from the HPCCC elution volumes and illustrated in Table 3-20.

Table 3-20: Comparison of K_D values of several target compounds between the two CCC separations

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Chitin adsorbate	0.16	0.26	0.77-1.08	0.57-0.97	3.52-4.34	3.93-5.76	5.97-6.07	0.48-0.74	0.26-0.77
C18 adsorbate	0.50-0.69	Not ident.	Not ident.	0.90-0.96	1.70-2.27	2.08-2.34	Not ident.	0.67-0.74	0.90-0.96

The specific experimental partition coefficients showed quite large variation between the two HPCCC runs of C18- and chitin- pigment adsorbates. Although these two separations were operated at identical conditions (flow rate, revolution, collection interval, temperature, etc.), their different metabolite composition resulting from different pre-purification methods might have caused variations in the sample specific intra- and inter- molecular forces between the structures, such as hydrogen-bond effects. It is known that hydrogen-bond effects could highly change the K_D values of metabolites showing dimer-formation as has been observed for protocatechuic acid and benzoic acid.[180,181] Because of different affinities to the solvent system, these samples might separate at very different elution times during CCC processing. Additionally, the unequal amount of injected sample (chitin sample was 1.25 fold higher than C18 sample) might affect the experimental elution volumes, especially when the samples contain highly polar compounds. That led to the differences in the breakthrough of mobile phase (until fraction 21 vs. 15) and carry-over of stationary phases in the two conducted runs (data not shown).

The resolution factor values $R_{i,j}$ of several target compounds were also calculated based on elution volume of specific compound similarly to the section 3.3.2.d for comparison of the separation power between two HPCCC runs. The results are displayed in the Table 3.21.

Table 3-21: Resolution factor values of several target compounds between the two HPCCC runs

R	$R_{1,2}$	$R_{2,3}$	$R_{3,4}$	$R_{4,1}$	$R_{4,5}$	$R_{5,6}$	$R_{6,7}$	$R_{8,9}$	$R_{1,8}$	$R_{4,9}$
Chitin extract	1.0	2.6	0.33	2.0	4.43	0.64	1.09	0.0	0.3	0.45
C18 extract	Not ident.	Not ident.	Not ident.	0.67	3.60	0.46	Not ident.	1.67	1.33	0.0

The $R_{i,j}$ value determination confirmed that the HPCCC approach fractionated the pigments and flavonoids of a pre-enriched extract and concentrated them into several fractions. For instance, celosianin II (**4**) had significant resolution values with both adjacent compounds of celosianin I (**3**) and quercetin-O-malonyl glucoside (**5**) ($R_{3,4}$: 0.33, $R_{4,5}$: 4.43). Although C18 adsorbate contained a lower number of target compounds than the chitin adsorbate, the HPCCC separation of the C18 was less efficient, resulting in lower $R_{i,j}$ between pigments and non-pigmented compounds compared to the chitin adsorbate ($R_{4,1}$, $R_{4,5}$, $R_{5,6}$). Nevertheless, the C18 extract separation resolved very well **8** and **9** ($R_{8,9}$, 1.67) which completely co-eluted in the chitin extract experiment. A similar result was found for **1** and **8** ($R_{1,8}$, 1.33 vs. 0.30) as well as the non-identified traces of *m/z* at 565 and 435 (data not shown). This resolution evaluation illustrated the different adsorption abilities of these two resins, in which chitin material has a much and very

specific stronger affinity to betalains. Chitin is a very selective approach to recover genuine betacyanins with three carboxy- functions. The results also suggest that the chitin pre-purification approach was highly suitable for the ion-pair HPCCC recovery of betalains on larger scale.

3.5.2.3 The studies of Atriplex flavonoids by HPCCC

a) LC-ESI-MS/MS screening of flavonoids purified by Sephadex LH20– K_D distribution ratio evaluation

Although 'chitin' and 'C18' purification adsorption approaches enriched betalains from *Atriplex* crude extract, these procedures could not concentrate effectively the flavonoids resulting in insufficient compound recovery for further investigation. A large Sephadex column was used as size exclusion chromatographic clean-up to recover the flavonoids from the ACN crude extract, in which a MeOH/water mixture was used. The partition distribution of the recovered flavonoids fraction was evaluated using several solvent systems available from literature,[180] resulting in the system of n-Hexane/n-BuOH/water (1/1/2) displaying satisfying distributions values for most of the non-polar and polar compounds (monitored by TLC). The distribution value K_D were quantitatively measured by LC-ESI-MS/MS and the chromatogram is shown in Figure 3-36.

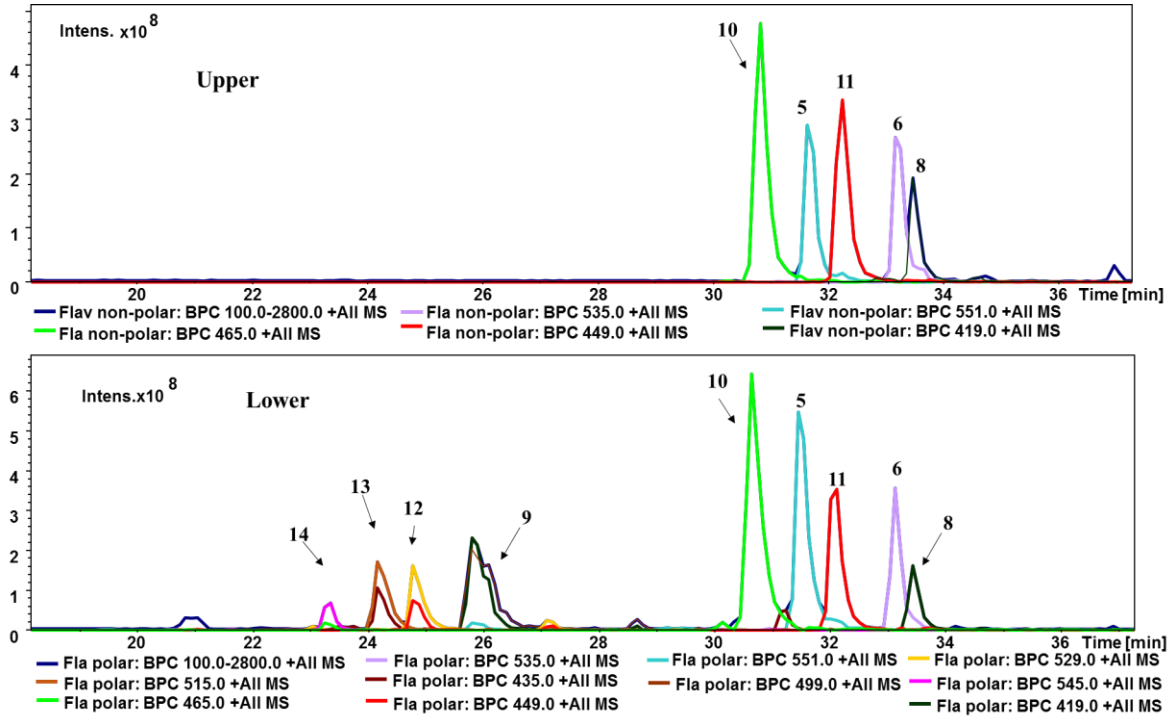


Figure 3-36: ESI-LC-MS analysis for determination of distribution ratio K_D of target flavonoids in upper and lower phases of the solvent system n-hexane/n-BuOH/$H_2$0 (1:1:2)

As it can be seen by the ion intensity values, most of the non-polar compounds **5**, **6**, **8**, **10**, **11** were distributed in the upper organic phase while parts of them remained in the more aqueous phase including the more polar compounds **9**, **12**, **13**, **14**. Therefore, a liquid/liquid partitioning extraction was done to initially fractionate these components into two samples namely upper (UP) fraction and lower (LO) fraction. For further solvent system evaluation, the K_D values of target compounds were calculated based on integrated peak areas elucidated from the ESI-LC-MS chromatogram of each fraction, using two different solvent systems n-hexane/n-BuOH/ water (1/1/2) for UP sample and and EtOAc/MeOH/ water (25/1/25) for LO sample. The results are presented in Table 3-22. Generally, the distribution values in the range from 0.5-1.5 which was suitable for HPCCC separation.[59]

Table 3-22: Distribution factor K_D evaluation for HPCCC of target compounds on non-polar and polar flavonoid fractions eluted from Sephadex LH20 column

	Non-polar fraction (UP) Solvent n-hexane/n-BuOH/ water (1/1/2)					Polar fraction (LO) Solvent EtOAc/MeOH/ water (25/1/25)			
Compounds	5	6	8	10	11	9	12	13	14
K_D	0.44	0.85	1.33	0.60	0.90	1.07	1.01	0.40	0.90

As expected, the elution orders of these compounds within two HPCCC separations (UP: injection 97 mg; LO injection 75 mg) were corresponding to their polarities, and were nearly identical to that of the ESI-LC-MS analysis. All collected fractions were monitored by TLC as fractions exhibiting similar spots were combined. The run of the non-polar sample (UP) led to 5 combined fractions namely UP1 (tube 18-19, 5.8 mg), UP2 (tube 20-28, 17.5 mg), UP3 (tube 29-33, 2.7 mg), UP4 (tube 48-66, 8.7 mg) and UP5 (tube 68-74, 2.7 mg). The separation of polar sample (LO) was less effective as the majority of target compounds were co-eluted within the first (combined) fraction LO1 (tube 12-18, 56.9 mg); whereas others fractions LO2 (tube 29-33, 0.8 mg), LO3 (tube 40-48, 1.1 mg), LO4 (tube 94-104, 0.2 mg) and LO5 (tube 107-110, 0.8 mg) contained only insignificant amounts. These fractions were then evaluated by ESI-LC-MS/MS and metabolite were identified by their UV spectra and MS/MS data. Fractions which appeared to be pure and recovered with adequate amounts were then structurally confirmed by 1D/2D-NMR measurements. These compounds were tentatively identified as flavonoid derivatives with different substitutions.

b) Identification of flavonoid glycosides by HPLC-DAD-ESI-MS/MS analyses

The HPLC-DAD-ESI-MS/MS data of combined fractions are summarized in Table 3-23, and were compared to those obtained with data of standard compounds in literature. The characteristic maximum absorbance at λ 340-360 nm and ESI-MS/MS data allowed the confirmation of several flavonoid constituents, which were poorly resolved by the HPCCC separations. These compounds generated the identical precursor ions $[M+H]^+$ at *m/z* 303 and 287 (positive mode), or $[M-H]^-$ at *m/z* 301 and 285 (negative mode) which matched the daughter fragmented ions of quercetin and kaempferol aglycones. The specific neutral loss differences characterized for linked sugar moieties, malonyl or sulphate groups which are commonly known to be attached to 3-O- and 7-O- positions of the aglycones.[182]

The compounds **5** and **10** were detected by $[M+H]^+$ mass signals at *m/z* 551 and 465 and both produced the fragment ion of quercetin backbone at *m/z* 301 (followed by *m/z* 179 and 151). The cleavages of Δ*m/z* 162 indicated a quercetin-hexoside (glucoside or galactoside). The additional neutral loss of Δ*m/z* 86 was found within the MS/MS of **5** spectra corresponding for the cleavage of a malonyl group.[183] That two compounds were tentatively identified as quercetin- malonyl-glucoside (**5**) and quercetin-glucoside (**10**).

Meanwhile, substance **14** displayed similar fragment data to **10** except the extra cleavage of 80 mass units representing for a sulphate group. The occurrence of sulphated flavonoids within a restricted number of plant families were known, particularly in the Chenopodiaceae family including this *Atriplex* genus. These flavonoid mono-sulphate esters could be recognized through the elimination of sugar ring $[M-H\text{-sugar}]^-$ and cleavage of SO_3 molecule $[M-H-80]^-$ from their MS/MS data. They were found more polar and eluted earlier than their parent flavonoid aglycones in the RP-HPLC.[184] That was relevant to the earlier elution of **14** compared to quercetin-glucoside (**10)** due to the extra linkage to the polar sulphate group; **14** was tentatively identified as quercetin sulphate glucoside.[185,186] Likewise, peak **13** also displayed the neutral losses Δ*m/z* 80 and Δ*m/z* 132 illustrating the cleavages of a mono-sulphate and a pentoside unit (xylose or arabinose), respectively. The spectrum of this compound **13** was found identical to that of quercetin 3-O-sulphate-7-O-α-arabino-pyranoside, which was previously reported from this *Atriplex hortensis*.[133] Figure 3-37 displayed the ESI-MS/MS spectra of these quercetin derivatives together with their tentatively suggested structures.

Table 3-23: LC-ESI-MS/MS analysis of flavonoid glycosides from *Atriplex* leaves found in HPCCC separation of two polar and non-polar fractions

Nº	Fractions	MW	R_t (min)	λ_{max} (nm)	$[M+H]^+$/ $[M-H]^-$/ ESI-MS^2	Tentative Identification
5	UP1; UP2 LO2	550	36.4	368	$[M+H]^+$: 551; MS^2: 302.7 (100), $[M-H]^-$: 549; MS^2: 300.9 (2.8), 505 (100), 463.9	Quercetin-3-malonyl-glucoside
6	UP2; UP3	534	39.7	368	$[M+H]^+$: 535; MS^2: 287 (100) $[M-H]^-$: 533, MS^2: 285 (9.6), 489 (100)	Kaempferol-O-malonyl glucoside
8	UP5 LO5	418	45.7	370	$[M+H]^+$: 419; MS^2: 287 (100) $[M-H]^-$: 417; MS^2: 285 (100)	Kaempferol-O-pentoside
9	LO1	498	35.5	368	$[M+H]^+$: 499; MS^2: 367(100), 287 (56,3) $[M-H]^-$: 497; MS^2: 417 (21.61), 285 (100)	Kaempferol -3-O-sulphate-7-O α-arabinopyranoside
10*	UP1; UP2; UP3; LO3	464	33.7	367	$[M+H]^+$: 465; MS^2: 302.9 (100). $[M-H]^-$: 463; MS^2: 300.9 (100), 179, 151	Quercetin-3-O-glucoside
11*	UP2; UP3; UP4; LO4	448	35.5	368	$[M+H]^+$: 449; MS^2: 287 (100) $[M-H]^-$: 447; MS^2: 285 (87.55), 254.7 (31.88)	Kaempferol -3-O-glucoside
12	LO1	528	25.5	369	$[M+H]^+$: 529; MS^2:449 (4.3),367 (100),287 (56,3) $[M-H]^-$: 527; MS^2: 447 (5.4), 285 (100)	Kaempferol- sulphate - glucoside
13	LO1	514	25.2	350	$[M+H]^+$: 515; MS^2: 302.7 (100), 382.8 (67.85) $[M-H]^-$: 513; MS^2: 300.8 (31.99), 432.9 (100)	Quercetin-3-O-sulphate 7-O-α-arabinopyranoside
14	-	544	32.4	266	$[M+H]^+$:545; MS^2: 302.7 (100), 382.8 (25.6), 464.9(7.2) $[M-H]^-$:543; MS^2: 300.7 (100), 342.8 (7.4), 62.9 (83.13)	Quercetin - sulphate - glucoside

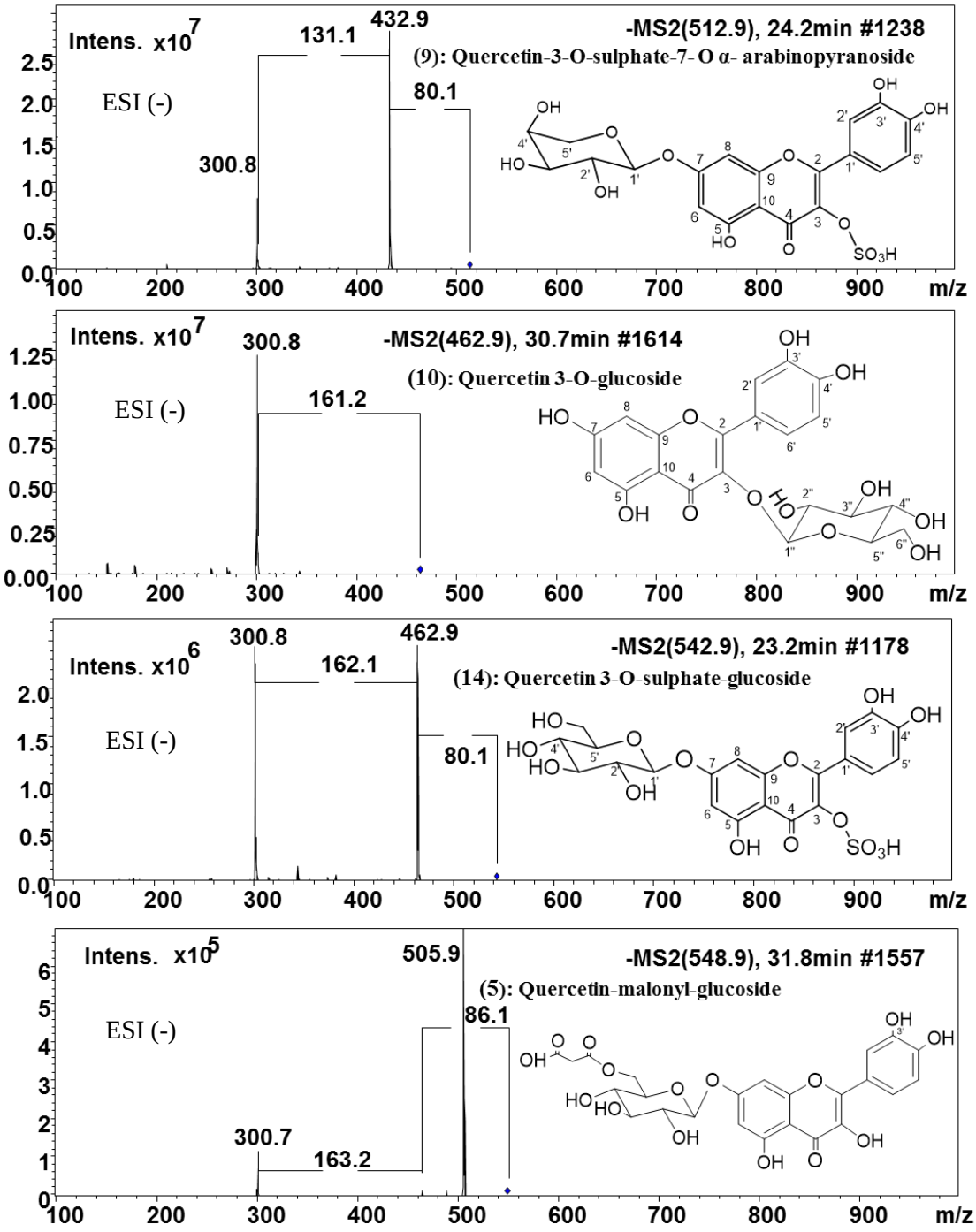


Figure 3-37: LC-MS chromatogram and suggested structures of quercetin derivatives from *Atriplex* ACN extract

Other metabolite **6**, **8**, **9**, **11**, **12** generated daughter ions at *m/z* 287 (positive ionization mode) or 285 (negative mode) indicating the flavonoid kaempferol, which were further characterized by the linkages of sugars, malonyl or sulphate moieties similarly to quercetin derivatives. The peaks **11** and **8** appeared with $[M+H]^+$ at *m/z* 449 and 419 (positive mode) and produced indicative aglycone fragment ions through the neutral losses of Δ*m/z* 162 (hexoside) and Δ*m/z* 132

(pentoside), respectively. These two compounds were tentatively identified as kaempferol pentoside (**8**)[187] and kaempferol glucoside (**11**). The 1D/2D-NMR structural confirmations were done for **10** and **11** due to their significant amounts recovered from both HPCCC runs. Likewise, the mass spectra of **6** included the cleavages of malonyl and hexoside units (positive ion mode), which was identical to that of kaempferol-malonyl-glucoside described in literature.[177] The co-existence of sulphated-kaempferol and sulphated-quercetin were reported previously for this genus, where kaempferol-3-O-sulphate-7-O-α-arabinopyranoside was also investigated by $^{1}H/^{13}C$ NMR and LC-MS.[133] The mass spectrum of **9** was found identically to that of sulphated-kaempferol-arabinopyranoside through the characteristic neutral losses of sulphate and the pentoside group. Additionally, its earlier elution on the LC-ESI-MS chromatogram confirmed the presence of the highly polar sulphate group compared to the kaempferol glucoside. Similarlily, **12** exhibited the molecular ion $[M+H]^+$ at *m/z* 529 (positive ion mode) and produced a fragment ion with a mass difference of Δ*m/z* 80. However, it revealed a hexose unit instead of a pentose compared to **9**. This compound was tentatively identified as kaempferol glucoside sulphate.[188,189]

Figure 3-38 illustrated the ESI-MS spectra of these kaempferol derivatives together with their suggested structures. These results were in agreement with the previous reports on the phytochemistry of the *Atriplex* genus, from which the glucosides of quercetin and kaempferol were found. However, isorhamnetin derivatives were not detected within this study.[133] Flavonoids are fundamental secondary metabolites found in many intensively consumed fruits and vegetables;[184] in which quercetin and kaempferol are the dominant structures including their various glycosides.

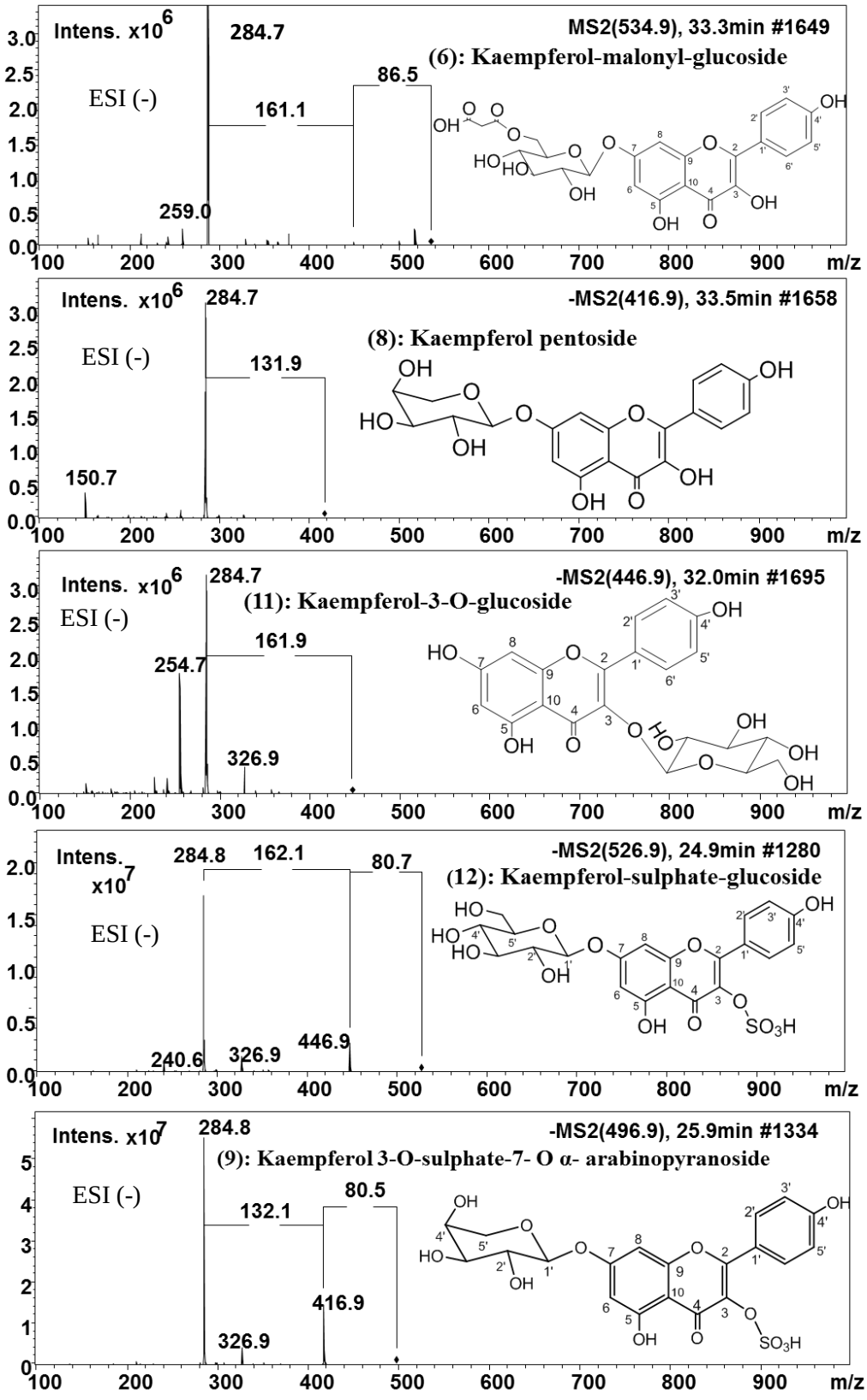


Figure 3-38: LC-MS chromatogram and suggested structures of kaempferol derivatives from *Atriplex* extract

3.5.2.4 Structural identification of the betacyanin Celosianin II and flavonoid glycosides by 1D/2D-NMR-spectroscopy from Atriplex hortensis var rubra

a) Celosianin II

1D-NMR (^{1}H, ^{13}C) and 2D-NMR (HSQC, HMBC, COSY, HSQC-TOSCY) were used to elucidate the structure of the most dominant pigment eluting from the ion-pair HPCCC separation of chitin enriched *Atriplex* adsorbate. This is the first time that 1D/2D NMR measurements were done on a high molecular weight betacyanin isolated from *Atriplex spp*. using the solvent mixture of ACN-d_3 with TFA-d_1 and D_2O. The ^{1}H and ^{13}C chemical shifts are summarized in Table 3-24, in which a few signals were overlapped and not clearly elucidated.

Table 3-24: ^{1}H and ^{13}C NMR data of (15*S*/15*R*)-Celosianin II (^{1}H: 700 MHz ^{13}C: 175 MHz, ACN-d_3/ TFA-d_1/D_2O: 98:1:1)

N^0	^{1}H(δ [ppm], mult, J [Hz])	^{13}C (δ [ppm])	N^0	^{1}H(δ [ppm], mult, J [Hz])	^{13}C (δ [ppm)	N^0	^{1}H(δ [ppm], mult, J [Hz])	^{13}C (δ [ppm])
2	5.2 dd (2.0,10.0)	64.6	**15**	4.56 dd(7.0,15)	53.2	**4’’**		n.d
3	3.33 m	33.8	**17**	-	149.4	**5’’**	n.d	n.d
	3.61 dd (9.0, 17.0)	-	**18**	6.43 s	106.0	**6’’**	n.d	n.d
4	7.12 s	111.9	**19**	-	n.d	**1’’’**	-	168.0
5	-	146.8	**20**	-	n.d	**2’’’**	6.31 d (15.4)	115.4
6	-	148.7	**1’**	4.87 d (8.0)	101.6	**3’’’**	7.55 d (15.4)	118.8
7	7.17s	100.1	**2’**	3.73	80.9	**4’’’**	n.d	n.d
8	-	138.0	**3’**	n.d	n.d	**5’’’**	n.d	123.6
9	-	125.6	**4’**	n.d	n.d	**6’’’**	n.d	n.d
10	-	n.d	**5’**	3.4	77.1	**7’’’**	-	146.8
11	8.37 d (12.2)	n.d	**6’**	3.64 m	62.2	**8’’’**	-	148.7
12	6.13 d (12.2)	110.8		3.78 m		**9’’’**	7.12	111.7
13	-	164.0	**1’’**	5.07 d (7.7)	101.1	**10’’’**	3.87 s	56.5
14	3.32m	27.7	**2’’**	3.39	77.4			
	3.45m		**3’’**		n.d			

(15*S*/15*R*)-celosianin II or betanidin 5-*O*-[2”-*O*-(E)-feruloyl-*β*-(1”,2’)-glucuronosyl- *β*-glucoside] is constructed by a glucosidic bond between betanin and *β*-D-glucuronic acid linked to E-ferulic acid. The chemical shifts and proton-proton, proton-carbon long range correlations together with their coupling constants were examined, showing accordance with the betacyanin NMR data reported before.[51] The betanidin structure was recognized by the identical singlets of H-4 (*δ* 7.12 ppm), H-7 (*δ* 7.17 ppm), H-18 (*δ* 6.43 ppm), the doublet signals of H-11, H-12 with each J 12.2 Hz at *δ* 8.37 ppm and *δ* 6.13 ppm together with three spin systems of H-2 (J 9.8 Hz, d, *δ* 5.19 ppm)/H-3a,b and H-14a,b/H-15.[190] The glycosylation at position C5 (*δ* 146.8 ppm) of betanidin with a *β*-D-glucose was indicated by different chemical shifts around *δ* 0.1 ppm at H-4 and H-7,[191] and the long-range H–C correlation between anomeric proton H-1’ (δ 4.87 ppm, d, J 8.0 Hz) and the C5 of the aromatic ring. The linkage at C2’ (*δ* 80.9 ppm) of the glucose unit with the aromatic C-1” (*δ* 101.1 ppm) of *β*-D-glucuronic acid was revealed through ^{2,3}J-CH correlation of H-1’’ (*δ* 5.07, d, 7.7 ppm) to C2’, together with the downfield shift of H2’ (*δ* 3.73 ppm) and the doublets of H1’ and H1”.[51] The glucuronic acid unit was connecting to E-feruloyl moiety at C2” (*δ* 77.4 ppm) represented by a low field acetylation shift of H-2” (*δ* 3.39 ppm). The singlet signal proton of a methoxy group and two doublet at H-2’’’ (*δ* 6.31 ppm, d, J 15.4) and H-3’’’ (*δ* 7.55 ppm, d, J 14.7) indicated the occurrence of E-feruloic acid. Unfortunately, the complete elucidation of the remained protons, carbons, and the H-C correlation of the glucoside and glucuronic acid were partly unresolved due to the co-existing of both 15*S*/15*R* stereoisomers and due to some impurities occurred by pigment instability. For instance, additional proton signals could be detected at H-4 and H-7 indicating for the pigment isomerization at low pH (pH around 2), or due to glycosidic bond hydrolysis, or the presence of pigment E-configuration displayed at C-12/C-13 double bond was shown by COSY (H-12, H-18; H-11, H-14). Nevertheless, the chemical shifts of the detected protons were in good accordance with published ^{1}H NMR data of this pigment generated by cell suspension cultures of *Chenopodium rubrum* before.[192,15] The structural relevant long range ^{2,3}J-CH correlations for Celosianin II are illustrated in Fig 3-39. To the best of our knowledge, these are the first ^{13}C and 2D-NMR data of celosianin II.

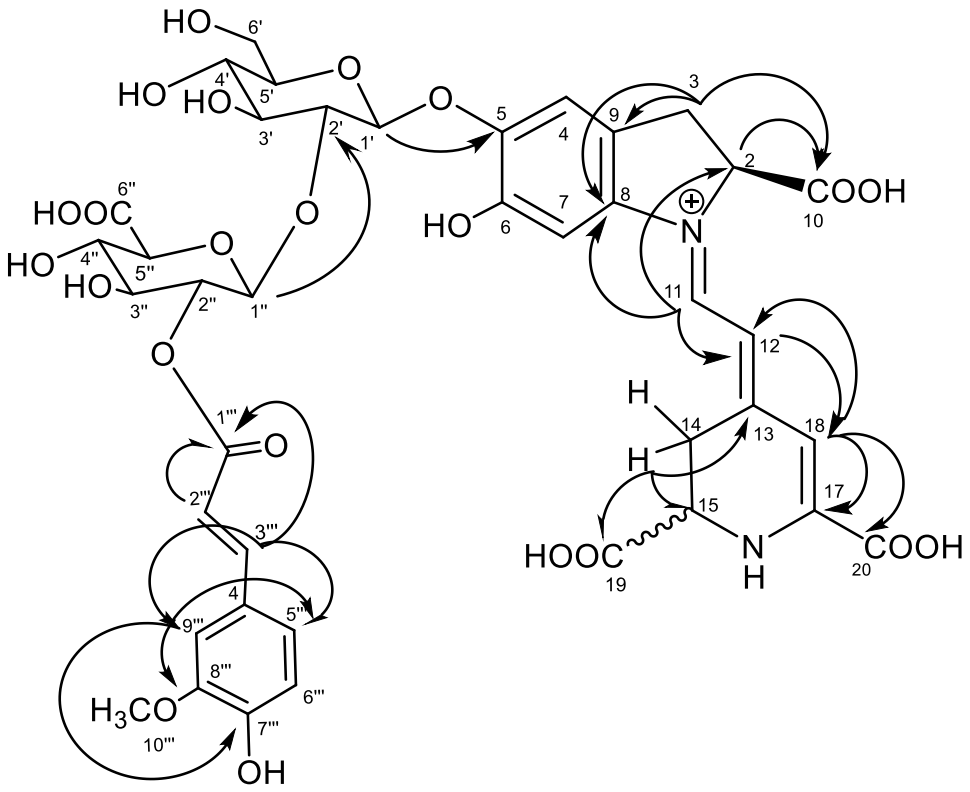


Figure 3-39: Celosianin II structure with important ^{2,3}J-CH-long-range correlation signals (700 MHz, ACN-d_3, TFA-d_1, D_2O)

b) Flavonoid glycosides from *Atriplex hortensis var rubra*

1D-(^{1}H, ^{13}C, DEPT-135) and 2D-NMR (HMBC, HSQC) experiments were performed for structural elucidation of flavonoid glycosides fractionated from two HPCCC separations. The combination of preparative column chromatography on Sephadex LH20 and the HPCCC on enriched extract resulted in only two pure compounds **10** and **11** that were measured in methanol-d_4. The chemical shifts from the ^{1}H and ^{13}C of both compounds are illustrated in Table 3-25. The ^{1}H NMR spectra of flavonoids and their glycosides were characterized by the set of aromatic protons, and the anomeric sugar signal with multiplets of oxymethin protons.

Table 3-25: ^{1}H and ^{13}C NMR data of Quercetin-3-O-glucoside and Kaempferol-3-O- glucoside

	Quercetin-3-O-glucoside		Kaempferol -3-O-glucoside	
	^{1}H δ [ppm], Mult., J [Hz]	^{13}C δ [ppm]	^{1}H δ [ppm], Mult., J [Hz]	^{13}C δ [ppm]
2	-	158.9	-	157.6
3	-	135.6	-	134.0
4	-	179.5	-	178.0

5	-	163.0	-	160.2
6	6.19 d (2.0)	99.8	6.20 d (1.9)	98.5
7	-	166.0	-	164.8
8	6.38 d (2.0)	94.7	6.41 d (1.9)	93.4
9	-	158.4	-	157.1
10	-	105.7	-	104.2
1’	-	123.0	-	121.4
2’	7.71 d (2.0)	116.0	8.07 AA’	130.9
3’	-	145.9	6.90 BB’	114.7
4’	-	149.8	-	161.7
5’	6.86 d (8.0)	117.5	6.90 AA’	114.7
6‘	7.57 dd (2.0, 8.0)	123.2	8.07 BB’	130.9
1”	5.26 d (7.5)	104.5	5.28 d (7.5)	102.6
2”	3.46 t (8.1)	75.7	3.45 m	74.3
3”	3.38 m	78.1	3.30m	76.6
4”	3.35 m	71.2	3.33 m	69.9
5”	3.22 m	78.4	3.22 m	77.0
6”	3.58 dd (5.2, 12.0)	62.5	3.58 dd (5.1, 12)	61.2
	3.72 dd (2.3, 12.0)		3.71 dd (2.3, 12)	

- Quercetin-3-O-*β*-D-glucoside:

The structural relevant long-range ^{2,3}J-CH correlations in the HMBC for this compound are illustrated in Figure 3-40, and the ^{1}H and ^{13}C NMR spectrum were compared with NMR data of the quercetin-3-*O*-β-D-glucoside available in literature.[193,180] The quercetin aglycone displayed the aromatic protons H-6 (*δ* 6.19 ppm, d, J 2.0 Hz) and H-8 (*δ* 6.38 ppm, d, J 2.0 Hz), and a typical coupling pattern H-2’(*δ* 7.71 ppm, d, J 2.0 Hz), H-6’(*δ* 7.57 ppm, dd, J_1 2.0 Hz, J_2 8.0

Hz) and H-5'(δ 6.86 ppm, d, J 8.0 Hz) of the B-ring system. The anomeric proton displayed at δ 5.26 ppm (J 7.5 Hz, H-1'') represented for β-configuration of the glucose moiety, and correlated to carbon of the aglycone at C3 (δ 135.6 ppm) elucidating the 3-O-glucoside linkage. The sugar moiety produced specific H6A" and H6B''proton signals with the chemical shifts at δ 3.58 ppm (J 5.2 Hz, 12.0 Hz) and δ 3.72 ppm (J 2.3 Hz, 12.0 Hz).

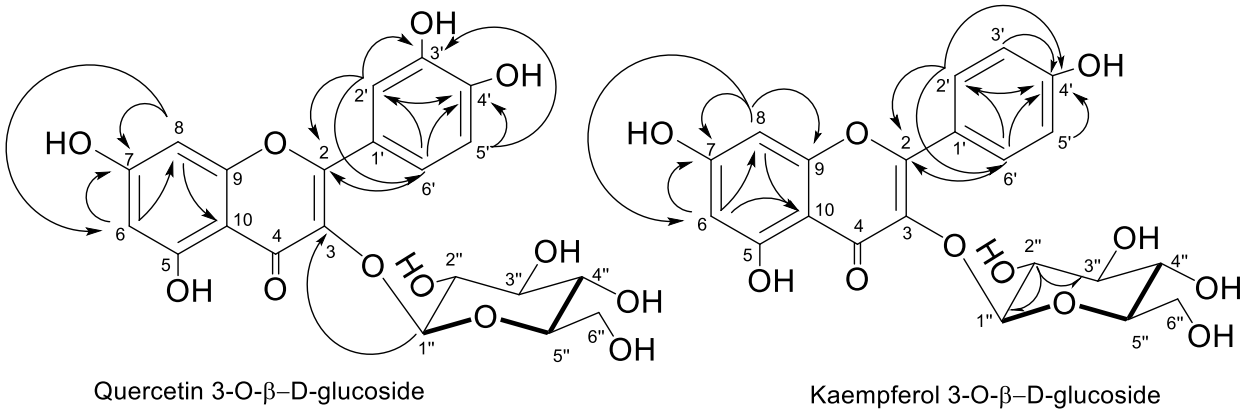


Figure 3-40: Quercetin and Kaempferol-3-O-β-D-glucoside structures elucidated by ^{2,3}J-CH long-range correlation signals from the HMBC

- Kaempferol 3-O-β-D-glucoside

The structural relevant long-range ^{2,3}J-CH correlations from HMBC of kaempferol-3-O-glucoside are displayed in Figure 3-40. In different to the spectra of quercetin aglycone, the kaempferol aglycone exhibited a sets of signals of a AA'-BB' coupling of the aromatic B-ring at δ 8.07 ppm (J 5.8 Hz) and and δ 6.9 ppm (J 7.0 Hz), assigning for the coupling of H-2',6' and H-3',5' respectively. The meta coupling aromatic protons H-6 (δ 6.21 ppm, J 1.9 Hz) and H-8 (δ 6.41ppm, J = 1.9 Hz) also proved the presence of that aglycone. The anomeric proton H-1'' appeared as a doublet at δ 5.28 ppm (J 7.5 Hz) in the ^{1}H, together with the ^{2,3}J-HC correlation of C3 in the HMBC indicated the attachment of a hexose at position 3-O of kaempferol. Likewise, the typical signals of glucose were recognized similarly to quercetin-3-O-glycoside at H6A" and H6B'' with J 12. The remaining ^{1}H/^{13}C signals were in agreement with the literature.[194]

3.5.3 Conclusions

To the best of our knowledge, this was the first report on the betalains and flavonoid constituents of *Atriplex hortensis* fractionated by HPCCC methodology. The method was able to fractionate pigments from most of the flavonoids glycosides, resulting in some pure fractions for structural elucidation by 1D/2D-NMR. These results confirmed the flavonoid profile of this *Atriplex spp*., particularly the presence of sulphated flavonoids together with pigments such as celosianin II. That makes the cultivar a promising candidate for pigment and flavonoid extraction.

4. SUMMARY: GENERAL CONCLUSIONS AND CONSIDERATIONS

This work developed a large lab-scale purification process for betalains using advanced HPCCC/CPC techniques with LC-ESI/MS/MS analysis or generation of sequential off-line ESI/MS/MS injection profiles of fractions, which has been applied for various of betalain plant sources. In order to obtain sufficient amount of isolated pigments for bioassay investigation or as reference standards for analysis, the process would use bio-competible and food grade chemicals. These results contributed to the previous reports on HPCCC separations of betalains from several pigmented cultivars such as *Opuntia*. *spp*, *Hylocereu*s *spp.*, *Atriple spp*; and confirmed the feasibility of large lab-scale isolation of pigments from these spieces. With the acquired knowledge a process scale-up for production-scale would be feasible.

Many food grade materials had been tested for affinity to betalains, resulting in the bio-polymer chitin/chitosan that showed a promising pigment binding capacity which was suitable for pre-purification process. The analytical comparison between chitin and chitosan adsorption abilities for betalains indicated that acidified chitin is having the highest binding capacity. A complete betalain pigments adsorption/desorption process using chitin was developed before trialed on some betalain materials such as *Beta vulgaris* (red beet) juices, *O.dillenii* (cactus pear), *Hylocereus polyrhizus* (dragon fruits), and *Bougainvillea glabra* bracts in order to evaluate the method productivities. This process was also applied in a fast and simple betaxanthin synthesis procedure to generate in-situ betalamic acid from purified betanin; from which vulgaxanthin I and indicaxanthin were synthesized successfully in a ‘one-step’ reaction.

The chitin process was applied for preparative enrichment of *Atriplex hortensis var rubra* pigments prior to the HPCCC separation. In this study the adsorption property of chitin was compared to that of reverse phase C18 resin, in which the pigments were pre-purified by chitin and C18 resin. Pigment extracts were introduced into HPCCC separations under similar operation conditions, and the recovery of fractions and separation quality were monitored by ESI-MS injection profiling method. The combination of chitin process with HPCCC monitoring by ESI-MS illustrated the superior selectivity of the chitin adsorption to betalains, from which celosianin II was isolated for structural elucidation by 1D/2D-NMR including ^{13}C NMR for the first time. Other nutraceuticals were available from this *Atriplex spp.* such as quercetin-3-O-glycoside, kaempferol-3-O-glycoside, kaempferol-3-O-sulphate-7-O-α-arabinopyranoside and quercetin-3-O-sulphate-7-O-α-arabinopyranoside. These compounds were also recognized through a Sephadex LH20 size-exclusion chromatography prior to the HPCCC purification. Although these flavonoids were not well resolved, these results contributed to the metabolite reports of this common leafly vegetable.

Likewise, chitin was also used to explore the pigment extract of *Hylocereus polyrhizus* fruits from Vietnam exhibiting intensive betalain contents; from which the minor pigments were better concentrated for HPCCC experiment. This study indicated the differences in pigment constituents of Vietnamese cultivar compared to others cultivars studied before, which are relevant to the distinct origination and cultivating conditions. The highly hydrophilic natures of these pigments such as betanidin 5-O-*β*-sophoroside, 2'-apiosyl-betanin, 2'-apiosyl-phyllocactin next to the typical structures of betanin, phyllocactin and hylocerenin, which required such strong ion-pair reagents such as heptafluorobutyric acid (HFBA) to enhance their equal distribution between two phases for a successful HPCCC separation.

The pigment extracts from *O. dillenii* were also introduced to HPCCC/CPC separation to investigate their metabolites and thermal stability under processing conditions. These scale-up experiments illustrated the comparable results between analytical and preparative runs on the same HPCCC spectrum under standard operating conditions; similar observations were seen in the comparison between HPCCC and CPC separation using the same C18 enriched pigment adsorbate. The analytical scale run showed as expected a better chromatographic resolution in the separation due to the lower concentration of sample, and observed K_D-values were identical to the preparative experiment. Meanwhile, the separation of heated pigments was less effective because of the changes in sample constituents caused by high amounts of thermal induced (degraded) products. These results illustrated the strong effect of sample compositions on HPCCC performances and that was in agreement with few reports on superior thermal resistances of *Opuntia* pigments such as the acylated phyllocactin. The heat resistances make this fruit a promising candidate for betalain extraction next to *Opuntia ficus* studied before.

Noteworthy, the combination of two techniques HPCCC and ESI-MS/MS profiling supplied general information about ionizable molecules present in each injected fraction; that allowed a direct identification of target compounds or impurities based on selected ion traces and MS/MS fragment data. Fractions which were detected to be pure could be submitted directly to 1D/2D-NMR structural elucidation, or go for further purification processes. However, this approach could not recognize the separation of stereoisomers (such as epimers betanin and isobetanin). They would require analysis in a second chromatography dimension such as HPLC or LC-MS. Within this study, no betalains isomer (15*S*/15*R* or *E*/*Z*) could be resolved.

Betalain biosynthesis is restricted to a minor group of plant families, from which only few of them are food plants where pigments and metabolites have been fully understood for food safety issues. The process developed within this study might suggest a simple approach for investigation of new betalain plant sources next to red beet and recently *Opuntia spp*. Nevertheless, more research will be needed to verify the pigment adsorption mechanism on

chitin/chitosan materials, and to examine the parameters effecting that binding capacity in order to maximize the pigment enrichment yields. Additionally, the betaxanthins synthesis using chitin could still be optimized and tested with others amino acids to fulfill the reaction mechanism next to reaction parameters. A specific synthesis of low concentrated betaxanthin could be done and time consuming isolation from plants could be omitted.

The potentials of HPCCC technique in preparative purification of betalains as well as other natural pigments are known. The uses of ion-pair perfluorinated carboxylic acids with the standard solvent systems TBMe/n-BuOH/ACN/water are incontrovertible since many efforts spent to replace these reagents led to no success. Solvents assisted by 0.7% TFA was able to fractionate the betalains from *Atriplex spp.* while strong acids as HFBA is needed for sufficient resolution of highly hydrophilic pigments from *Opuntia spp.* and dragon fruits. However, strong reagents as HFBA are not encouraged in betacyanic pigment isolation because of their high persistence, they remain as toxic reagents in the collected fractions afterwards. The extremely polar and unstable nature of betalains still makes the search for suitable CCC solvent systems very challenging.

5. MATERIALS AND METHODS

5.1 MATERIALS

Solvents, chemicals and reagents used in this study are summarized in Table 5.1.

Table 5-1: List of solvents, chemicals and reagents used in this study

Chemicals	Quality	Manufacture
Acetonitrile	HPLC grade	Honeywell (Seelze, Germany)
Acetonitrile	LC-MS grade	Honeywell (Seelze, Germany)
Acetonitril-d_3	99.96 %,	Deutero (Kastellaun, Germany)
Acetic acid	p.a. grade, ≥99.8%	Sigma (Steinheim, Germany)
Ammonium acetate	97%	AppliChem GmbH, Darmstadt, Germany
Ammonia solution	25%	VWR International S.A.S. Fontenay-sous-Bois
C18 resin for liquid chromatography	40-63 µm	Merck KGaA, Darmstadt, Germany
Chitin	p.a. grade	Nha Trang University, Vietnam
Chitosan	p.a. grade	Cognig GmbH, Dusseldorf, Germany
Dichloromethane (DCM)	HPLC grade	Fisher Scientific (Loghborough, UK)
D_2O	99.95% D	Deutero (Kastellaun, Germany)
Ethanol	HPLC grade	Sigma (Steinheim, Germany)
Ethyl acetate	HPLC grade	Sigma-Aldrich, St. Louis, Missouri, Vereinigte Staaten
Formic acid	LC-MS grade	Sigma-Aldrich (Deisenhofen, Germany)
Iso-propanol	HPLC	Fischer Chemicals Pittsburgh, USA
Lipophilic Sephadex ®	25-100µ	Sigma-Aldrich, St. Louis, (Steinheim, Germany)
LiChroprep® RP-18 for LC	40-63µ	Merck KGaA, Darmstadt, Germany
L-glutamic acid	≥ 98.5%	Carl Roth, Karlsruhe - Germany

Chemicals	Quality	Manufacture
L-prolin ≥ 98.5%	≥ 98.5%	Carl Roth, Karlsruhe - Germany
Methanol	HPLC grade	Fischer Chemicals Pittsburgh, USA
Methanol-d_4	99.95% D	Deutero (Kastellaun, Germany)
NANOpure Diamond Analytical	0.2 µm	Wilhelm Werner, Leverkusen, Germany
n-Butanol	HPLC grade	Carl Roth (Karlsruhe, Germany)
n-Hexane	HPLC grade	Fisher Scientific (Loghborough, UK)
p-Anisaldehyde	>99%	Fisher Scientific (Loghborough, UK)
Paper filter	MN 165 ¼, ϕ 500	Macherey-Nagel, Düren, Germany
pH paper	pH 1-11	Macherey-Nagel, Düren, Germany
Propionic acid	LC-MS	Sigma-Aldrich, St. Louis, Missouri, Vereinigte Staaten
Silica gel 60 TLC plates	F254	Merck KGaA, Darmstadt, Germany
C18 plate	RP-18W/UV_{254}	Macherey-Nagel, Düren, Germany
Trifluoro acetic acid (TFA)	99%	Sigma-Aldrich, St. Louis, (Steinheim, Germany)
Trifluoro acetic acid (TFA-d_1)	99.95% D	Deutero GmbH, Kastellaun, Deutschland
Tetramethylsilan (TMS)	NMR-grade	Sigma-Aldrich, St. Louis, (Steinheim, Germany)

Betalains materials used within this studies are described in Table 5.2. All materials were frozen and stored below -20^0C at Institute of Food Chemistry of the TUBraunschweig during the experiments.

Table 5-2: List of betalainic materials used in this study

Materials	Parts	Originate
Red beet (*Beta vulgaris*)	Fresh roots	Local market (Braunschweig,Germany)
Thermal treated red beet	Juices	Super market (Braunschweig,Germany)

Opuntia stricta var dellini	Frozen fruit	Egypt
Hylocereus polyrhizus (Red dragon fruit)	Fresh fruits	Vietnam
Bougainvillea glabra	Bracts	Mexico
Atriplex hortensis var rubra	Leaves	Braunschweig, Germany

5.2 METHODS

5.2.1 The adsorption/desorption of betalains by chitin/chitosan

5.2.1.1 Pigments extraction

All materials were blended by a kitchen blender before being macerated several times with the ACN:Nanopure water 0.7% acetic acid (AcOH) mixture. The fruit/root materials (*Opuntia*, *Hylocereus polyrhizus* and red beet roots) were extracted with ACN:water (7:3, v:v) while leaves/flower materials (*Atriplex spp*. and *Bougainvillea bracts*) were done with ACN:water (1:1, v:v) mixture.

After 15-30 min of incubation, the extracts were filtered out by paper filter with large funnels to remove the residues and then directly lyophilized by a freeze drier (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The dried extracts were kept in the dark at -20^0C for further analysis. All extraction processes were done as fast as possible under light protection and at room temperature to limit betalain pigment degradation.

5.2.1.2 Chitin/chitosan powder preparation

The chitin/chitosan fine powders were washed several times with Nanopure water to remove the soluble contaminants and residual soluble salts until the supernatant was clear and a neutral pH was reached. The resins were then activated with 0.7% AcOH for 1 hour, then the water was decanted and the wet chitin/chitosan solids were used for the adsorption experiments.

5.2.1.3 Pigment adsorption and desorption

The dried pigments extracts were dissolved in the desired concentration before being mixed with activated chitin in a beaker for two hours. The pigmented slurry was then washed several times with Nanopure water 0.7% AcOH until the supernatants were clear and colorless. These supernatants were also monitored by TLC on silicagel plates and visualized by anisaldehyde

reagent at 105^{o}C until the green/brown color was gone, which indicated the sugars were completely washed out.

For desorption, the pigmented chitin/chitosan resins were incubated with EtOH:0.15M ammonium acetate (AmAc) (v:v, 25:75). This desorption was repeated several times to recover most of the adsorbed pigments until the pigment intensity was pale. The supernatants were filtered and directly freeze-dried for further analysis. The lyophilization were repeated several times until the AmAc sublimated completely.

The comparison of adsorption capacities amongst chitin/chitosan/acidic treated chitin was undertaken following the procedure of Wang et al. (2017) with minor modifications: 10 ml of *Hylocereus* pigment solutions (containing approximately 0.305 mg of betacyanin) was added to 1g of activated chitin/chitosan and incubated for 1 hour; the supernatant was then filtered and measured by UV-Vis spectrophotometer at λ 535 nm.

The adsorption/desorption of betalains pigments from other betalains plant materials on acidic chitin was done similarly to investigate the process reproducibility. The supernatant of each experiment was quantitatively analyzed by UV-Vis spectrophotometer at λ 535 nm, and qualitatively LC-ESI-MS analysis for comparison of pigment constitution and profile changes.

5.2.2 Betaxanthin synthesis

The chitin powder was introduced into a 24 cm x 3 cm column (Aldrich), and activated by washing several times with aqueous AcOH. 5 ml of commercial red beet juice was added gently to the columns for betanin adsorption, followed by multiple washing steps until the eluted water was colorless and free of soluble products such as sugars (monitored by TLC). 2M ammonium hydroxide solution was added (30 ml) to hydrolyze the adsorbed betanin. The pale yellow eluted solution was dropped directly to a solution of a dissolved amino acid (cf. Figure 3-13) acidified with concentrated AcOH (pH around 5-6) followed by vigorous shaking until the colors changed from pale green/ yellow to red/ orange.

5.2.3 Larger scale extraction, enrichment of *Opuntia* pigments/non-pigmented compounds

5.2.3.1 Pigment extraction and enrichment by reversed phase C18 materials

Pigment extraction: The whole frozen fruits were processed by a home-style juicer (Philips juicer HR1861, 700W) and filtered through a cotton cloth and then paper filter. The residuals including flesh, seeds and peels were washed several times with water; juices were combined for the enrichment step by C18 reversed phase resin.

Pigment enriched by reversed phase C18 material: C18 solid was washed three times with ACN, conditioned with aqueous acetic acid (0.7%) four times before being incubated with the *Opuntia*

crude extract for 1h in a large beaker. The pigment adsorbed resin was rinsed exhaustively with aqueous acetic acid (0.7%) to remove unadsorbed compounds such as sugars and fruits acids (monitored by TLC). For pigment desorption, the colored resin was incubated 1hour with EtOH:water (2:8, v:v, 0.7% acetic acid); the supernatant was filtered and freeze-dried. The desorption procedure of the resin was done several times until the purple color was significantly reduced.

5.2.3.2 Non-pigment extraction and enrichment by Sephadex large column size exclusion chromatography

The fruit residues (seeds, peels, flesh) were extracted several times with dichloromethane. The extract was filtered and concentrated by a rotary evaporator (Heidolph Instruments GmbH & Co. KG, model: Laborota 4000, Schwabach, Germany) at minimum temperature 40^{o}C under reduced pressure 50 mbar before enrichment by a Sephadex chromatography.

Sephadex size-exclusion chromatography: The concentrated extract (7.5g) was then applied onto a Sephadex column (length 1.5 m, diameter 7 cm). The compounds were eluted by MeOH and monitored by both TLC and ESI-MS/MS injection profiling to specify the trace compounds of interest.

The fractions containing unknown target compounds (*m/z* 620, 602, 604) were combined and freeze dried. Approximately 3 g of the dried fraction were dissolved with MeOH:water (v:v, 1:1) following by the liquid –liquid partitioning extraction (5 times) with dichlomethane. The resulted partitions (upper and lower phases) were evaluated by TLC and LC/ESI/MS examination. The target compounds were mostly concentrated in the organic phase (around 700 mg) which was then separated by a HPCCC approach.

5.2.3.3 Thermal treatment of Opuntia (C18) enriched pigment

The dried C18 pigment extract (500 mg) was dissolved with nanopure water and treated at 85^{o}C for 1 hour by a reactive vial within a heat block. The heated sample was then cooled to ambient temperature before freeze drying and stored for further HPCCC separations.

5.2.4 HPCCC apparatus and operation

5.2.4.1 HPCCC apparatus

The analytical and semi-preparative separations were carried out on a multilayer coil planet J-type Spectrum HPCCC (Dynamic Extractions, Gwent, UK) with highest operation speed of 1600 rpm. The device has two-connected semi-preparative coil columns (63 mL + 62.5 mL, 1.6 mm tube i.d.) and two-connected analytical coil columns (11 mL + 11.5 mL, 0.8 mm tube i.d.) which

are made of polytetrafluoroethylene tubing and mounted on a two-bobbin system. The revolution radius R is 85 mm with a β value range of 0.64-0.81 and 0.52-0.86 for analytical and preparative coils, respectively.

The operations temperatures were kept constant at 30^{o} C by an external cooling system. The solvents were delivered by a preparative LC pump (K-501 from Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany). The eluted fractions were collected in the interval of 1 min/tube by a fraction collector of Pharmacia Superfrac (Uppsala, Sweden) and the UV detection at λ 540 nm (K-2500 Knauer Wissenschaftliche Geräte).

5.2.4.2 HPCCC operation

The solvents were added into a separatory funnel at room temperature and shaken vigorously to equilibrium before left to form two clear layers; the upper layer (stationary phase) and lower layer (mobile phase) were ultra-sonicated shortly before operation. The '*head to tail*' approach and '*elution – extrusion'* mode were applied for all the separations, in which the coil system was fully filled with stationary phase before starting the maximum rotation speed of 1600 rpm. The mobile phase was then delivered to the system at desired flow rate until the hydrodynamic equilibrium reached, and the volume of displaced stationary phase was measured for calculating the stationary phase retention value (S_f). The sample was dissolved in equal amounts of upper and lower phases, sonicated, filtrated (Chromafil Xtra GF-100/25) then injected into a 5 ml injection loop by a plastic syringe. When the operation temperature was stable at 30^{o} C, the sample was introduced into the system through a manual low-pressure injection valve (Rheodyne, Cotati, CA, USA). After the mobile phase passed two coil volumes, the *extrusion* mode was started by changing the stationary phase at desired flow rate and interval collection time accordingly. The elutions runs were finished after over one-coil volume of stationary phase was collected followed by extrusion performance.

5.2.5 CPC apparatus and operation

A large-scale FCPC (Kromaton Technologies, Angers, France, with a 200 ml rotor) was used. The system was operated in *descending* mode in which the solvent was pushed from the top to the bottom of each separation chamber. The speed was set to medium velocity at 1000 rpm, flow rate was 5.0 ml/min which was delivered by L-6250 Intelligent pump (Merck-Hitachi, Tokyo, Japan) and collected fraction was 1 min/tube (Pharmacia Superfrac, Uppsala, Sweden). After the hydrodynamic equilibrium was reached, the sample was dissolved in 2.5 ml upper phase + 2.5 ml lower phase, sonicated and introduced into the system by a 10 ml sample loop. The elution was finished at tube 50, the mobile phased was changed to stationary phase for extrusion. The operation was finished after more than one coil volume of extrusion.

5.2.6 Metabolite profiling by *off-line* ESI-MS/MS injection

For every HPCCC separation, an aliquot amount of each collected fraction was transferred into a HPLC vial before sequentially injected to the ESI-MS/MS system by an auto-sampler device (AS-2000A, Merck-Hitachi, Tokyo, Japan). The sequential injection was started from the fraction where compounds started eluting after the breakthrough of mobile phase. For each vial, the same volume was injected within the time interval of 2 minutes; the make-up solvent was delivered at flow rate of 0.5 mL/min using a binary HPLC-pump (G1312 A, 1100 Series, Agilent, Wald-bronn, Germany). To keep the relevant ion intensity of single peak within each ESI-MS injection profile in an adequate level and to avoid the overloading of highly concentrated fractions to the ESI-MS source, fast pre-injecting experiments of some fractions were done according to the initial concentration of each separation. A suitable ion intensity was around 1.0×10^6-10^8 for base peak signals.

ESI-MS/MS parameter settings: alternating ionization mode in the scan range of *m/z* 100–2000; the "ultra"-mode with a mass scanning rate of 26.000 *m/z* per second was chosen. Drying gas was nitrogen (10.0 L/min, 320°C) and nebuliser pressure was 60 psi. High ionization voltage (HV) capillary was -3500 V, HV end plate offset -500 V, trap drive 79.2, octopole radiofrequency (RF) amplitude 187.1 Vpp, lens 2 60.0 V, Cap Ex 115.0 V, maximum accumulation time 200 ms. Averages of five spectra, trap drive level 120%, target mass range: *m/z* 500, compound stability 80%, Smart ICC target 100000, ICC charge control "on" and smart parameter setting "active". 5-7 most intensive precursor ions were selected for fragmentation. The remaining MS/MS fragmentation amplitude value was 1V.

For the separations of *Opuntia* pigments, an aliquot volume of 300µL of each fraction was diluted with 1ml of make-up solvent ACN:water:formic acid (v:v, 50:50:1%) together with 20 µl of propionic acid within each single vial. The analytical profile was split into two injection experiments in which the injected volumes of the non-pigmented compounds were adjusted because of the high pigment concentration in the initial fractions (intensity 10^8 vs. 10^7). The remaining fractions were mixed with nanopure water and freeze-dried for further investigation.

For HPCCC separation of *Atriplex* metabolites, the ESI-MS profile was also processed using ACN:water (1:1, v:v) as make-up solvent. Injection volume was 20 µL. The remaining compounds of all fractions were dried by Speed-Vac concentrator (Thermal Savant RVT400 Refrigerated Vapor Tap, Waltham, USA) for further investigation.

5.2.7 Thin layer chromatography (TLC)

TLC was used to monitor the purities of the pigment extract and the fractions being eluted from HPCCC; for detection, the TLC plate was sprayed with anisaldehyde-acetic acid reagent (5 mL

anisaldehyde + 100 mL acetic acid + 10 mL sulphuric acid and acetic acid up to 500 ml) and heated at 105ºC.

Depending on the extracts, RP C18 or silicagel plate were used with the corresponding solvent system. Table 5.3 summarizes all the TLC experiments applied on each sample and solvent system.

Table 5-3: Summary all the TLC experiments applied on each sample and solvent system

Plate	Sample	Solvent
C18	sugars or acids remained from pigment extract	
C18	non-polar compounds of *Opuntia*	ACN/water (8/2, v/v)
Silicagel	non-polar compounds of *Opuntia*	DCM/MeOH/water (75/25/1, v/v/v)
Silicagel	non-polar compounds of *Atriplex*	DCM/MeOH/water (75/25/1, v/v/v) EtOAc/MeOH/water (30/5/4, v/v/v)

5.2.8 Betacyanins quantitative analysis by UV-Vis spectrophotometer

Spectrometer: The UV-Vis spectrophotometer V-570 Jasco (Gross-Umstadt, Germany)

λ: 535nm

Software: Spectramanager v1.27.02

Statistical analysis: Statistical Package for the Social Sciences software (SPSS)

5.2.9 Pigments analyzed by LC-ESI-MS/MS

Solvent A: water 1% HCOOH; solvent B: ACN 1% HCOOH

Column: Prontosil C18-Aq column (250mm× 2.0mm, 5 μ, 100 Å, Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany)

Ion-trap-electrospray mass-spectrometer: HCT-Ultra ETD II Bruker Daltonics (Bremen, Germany)

Pump: Binary HPLC-pump (model G1312 A, 1100 Series, Agilent Technologies, Waldbronn, Germany)

Autosampler and detector: ALS-SL G1329B, FC/ALS therm G1330B, Agilent Technologies (Waldbronn, Germany).

Software: Data analysis version 3.0 (Bruker Daltonics, Bremen)

Linear gradient set up:

Time (min)	0	20	35	45	55	60	70
A (%)	99	80	50	0	0	99	99
B (%)	1	20	50	100	100	1	1

ESI-MS/MS parameter settings:

Alternating ionization mode in the scan range of *m/z* 100–1500; the "ultra"-mode with a mass scanning rate of 26.000 *m/z* per second. Drying gas was nitrogen (11.0 L/min, 330°C) and nebuliser pressure was 60 psi. High ionization voltage (HV) capillary was -3700 V, HV end plate offset -500 V, trap drive 72.1, octopole radiofrequency (RF) amplitude 187.1 Vpp, lens 2 60.0 V, Cap Ex 115.0 V, maximum accumulation time 200 ms. Averages of five spectra, trap drive level 120%, target mass range: *m/z* 500, compound stability 80%, Smart ICC target 100000, ICC charge control "on" and smart parameter setting "active". 3 most intensive precursor ions were selected for fragmentation. The MS/MS fragmentation amplitude value was 1V.

The samples was diluted with water at concentration of 0.5-1 mg/ml and injected volume ranged from 10 – 15 µL depending on the concentration.

5.2.10 Lyophilization, evaporation of fractions collected from HPCCC experiments

The pigmented fractions were recovered within the elution-mode. Aqueous solutes were completely frozen before freeze drying (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The organic fractions (non-polar fractions) eluted mostly in the extrusion-mode and were concentrated by a SpeedVac Plus concentrator equipped with a rotor for tubes (*SC210A*), and refrigerated vapor trap (RVT 400, Thermo Savant, Holbrook, NY, USA). The dried fractions were then weighted.

5.2.11 Structural elucidation of purified compounds by NMR

The identification of flavonoid glycosides isolated from *Atriplex hortensis var rubra* was done by 300 MHz NMR spectrometer (Bruker FT 300, Bruker Biospin, Rheinstetten, Germany) usng methanol-d_4 while 1D/2D-NMR experiments of the pigment celosian II was measured on a 700 MHz NMR spectrophotometer using the solvent of ACN-d_3, TFA-d_1, D_2O (98:1:1, v:v). Software was TopSpin, Bruker.

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